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DISEASES OF CHILDREN

PART II

Symposium on Amaurotic Family Idiocy (Tay-Sachs' Disease)

COCHAIRMEN: BRUNO W. VOLK, M.D.
W. D. SPERRY, Ph.D.

Held at the
Isaac Albert Research Institute of the
Jewish Chronic Disease Hospital, Brooklyn, N. Y.

Thursday, April 10, 1958

Under the auspices of the
Albert Einstein College of Medicine
of Yeshiva University and the
National Tay-Sachs Association, New York

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Journal of Diseases of Children

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A.M.A. JOURNAL OF
DISEASES OF CHILDREN

Introductory Remarks, Morning Session

BRUNO W. VOLK, M.D.

Except for the minor changes necessary to adapt them to the typography of the A. M. A. JOURNAL OF DISEASES OF CHILDREN, *the papers in this symposium are published exactly as received, and neither the Editorial Board of the* JOURNAL *nor the manuscript editing section of the American Medical Association can assume responsibility for their editorial style.*

On behalf of the staff of the newly inaugurated Isaac Albert Research Institute, I wish to welcome the participants in this symposium. There are present, today, interested and dedicated scientists from many research centers in this country and abroad who will discuss their findings concerning the lipidoses. It is our mutual hope that this interchange of ideas will serve to bring closer the day when this group of diseases will no longer be a menace.

The study of amaurotic family idiocy (Tay-Sachs' disease) and allied disorders at this hospital started about six years ago when the first baby afflicted with the infantile form of this disease was autopsied. Since then more than 111 cases of Tay-Sachs' disease have been analyzed and many of the patients hospitalized for extended periods of observation. The clinical observations as well as anthropometric and pneumoencephalographic studies were correlated with serial biochemical data of blood and cerebrospinal fluid, including the determinations of proteins and their subfractions, various enzymes, lipids and neuraminic acid. When autopsies were performed the morphologic findings within the central nervous system were coordinated with the biochemical changes occurring in these tissues.

The multiphasic approach to the study of amaurotic family idiocy made possible through the benevolent cooperation of the hospital administration led to the recent establishment of a ward dedicated to the care of children afflicted with this disease and allied disorders such as Niemann- Pick's disease, Gaucher's disease and Schilder's disease.

The members of the Isaac Albert Research Institute are most gratified that the Albert Einstein College of Medicine of Yeshiva University and the National Tay-Sachs' Association have seen fit to sponsor this Symposium on Tay-Sachs' Disease which is held in the newly erected laboratories of this Institute. Its purpose is to explore the difficult aspects of this problem, to exchange concepts of various investigators and to discuss newer work which may result in a better understanding of this tragic condition and no doubt stimulate additional studies. It also may help parents of children fflicted with this disease to overcome their feeling of isolation and helplessness. It may encourage them to join hands in promoting studies concerning the sociologic aspects of this disease, an undertaking which of necessity will supplement the work of the professional investigators. It is thus hoped that these conjoint efforts will eventually lead to a better understanding of the nature of Tay-Sachs' disease and equally contribute to a solution of other allied degenerative diseases involving the central nervous system.

Isaac Albert Research Institute, Jewish Chronic Disease Hospital, Brooklyn.

Clinical Progression of Amaurotic Family Idiocy

Anthropometric Studies

ABRAM KANOF, M.D., STANLEY M. ARONSON, M.D., and BRUNO W. VOLK, M.D.

The data upon which this presentation is based consist of continuing and protracted observation of 111 patients with Tay-Sachs' disease and Niemann-Pick's disease (Table 1). Twenty-eight of these patients were hospitalized at this institution for extended terms, and of these, 23 have died and 21 have been autopsied.

The studies herein summarized arose from initial observations four years ago, on a single patient with Tay-Sachs' disease. After 15 months of institutionalization, there suddenly began a progressive cranial enlargement. This seemed contrary to the anticipated evolution of a disease which is generally characterized by arrest of development. On the basis of this observation additional children were admitted, and again a comparable head enlargement was noted. This paradoxical increase in head circumference did not occur early in the disorder but appeared during the later stages of the disease.[1]

Since the initial observations, a total of 28 children afflicted with Tay-Sachs' disease have been serially studied with periodic pneumoencephalograms as well as weekly recordings of the head and chest circumferences and body weight. In some cases, periodic roentgenograms of the epiphyses were taken to determine bone age. In addition to the parameters mentioned above, pathologic data on most of these children were ultimately available. In accordance with general experience in the past, the vast majority of our patients were of Jewish extraction. Most of the grandparents of the afflicted children were born in the northeastern zones of Europe. Only four were of non-Jewish parentage.

Obstetrical data of the children showed no significant variations during gestation. Birth weights were within normal limits, and generally speaking the first few months of existence were described as normal by the parents. Symptoms usually commenced between the third and the tenth months of life, the onset averaging approximately 5.5 months (Table 3). Initial signs believed to be directly attributable to the underlying disorder were quite varied but could be classified generally as neuroirritative or neurodepressive. The former were more characteristic of the early stages of the disease and consisted generally of hypersensitivity to sound, and to a lesser degree, to light. As the disease progressed, epileptiform symptoms were almost constantly

From the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn.

TABLE 1.—*Central Nervous System Lipidoses*

Diagnosis			Number
Amaurotic Family Idiocy.	male	60	
	female	48	
			108
Niemann-Pick's Disease.	male	3	
	female	0	
			3
Total Cases Studied			111

TABLE 2.—*Foreign Countries of Origin, Grandparents of Children with Infantile Amaurotic Family Idiocy* *

Russia, Poland, Baltic States:	72.7%
Austro-Hungarian Empire:	22.3%
Germany:	2.3%
France, England:	2.3%
Middle East Countries:	0.4%

* Cases in which grandparents were born in the United States and cases where place of birth was obscure, have not been included.

TABLE 3.—*Onset and Duration of Determinable Disease in 108 Cases of Infantile Amaurotic Family Idiocy*

Onset		Duration	
Month	No. Cases	Month	No. Cases
Birth	2	0-12	3
1	2	13-18	14
2	6	19-24	20
3	13	25-30	16
4	14	31-36	9
5	19	37-42	8
6	14	43-48	2
7	16	49-60	3
8	10	61+	0
9	5		—
10	7		75 *
	—		
	108		
Average: 5.5 months		Average: 25.9 months	

* The remaining 33 children are either still alive, or date of death not accurately known.

TABLE 4.—*Initial Symptoms in 73 Cases of Infantile Amaurotic Family Idiocy*

Symptom	No. Cases *
Hyperacusis	37
Sensitivity to light	1
Abnormal limb movements	7
Abnormal eye movements	4
Abnormal head movements	2
Generalized seizures	3
Could not sit up	17
Could not hold head up	9
Could not turn over	5
Weakness, generalized	6
Listlessness, lack of awareness	10
Lack of any movement	1
Strabismus	1
Irritability	1
Failing vision	3
Feet "turned in"	5
Feeding difficulties	1
Insomnia **	2

* In some of the cases, the parents described more than one abnormality as the initiation of the illness.

** Probably a manifestation of hyperacusis.

noted. The neurodepressive component of the disease was principally manifested by progressive paresis ultimately leading to total quadriplegia and an immobile, vegetative existence (Table 4). All the children had macular degeneration.

The onset of a diffuse neurologic disorder during the time of maximal somatic maturation has resulted in one of the principal characterizations of the disease originally described by Sachs himself, namely, arrest of development. As the disease pursued its course, it became apparent that there was an increasing retardation of growth in all dimensions. An apparent reversal of this retardation in regard to the cranium occurred after approximately 18 months of illness. Such megalencephaly has not been described with this disease in the past, principally because survival for periods beyond 18 months of illness has been most infrequent. The protracted clinical course exhibited by many of the children was due principally to the judicious use of antibiotics, constant aspiration, careful nursing supervision and an overall conscientious effort to prolong the existence of these children. No specific therapeutic agents were used.

For purposes of study, the disease has been subdivided into three phases, the characteristics of which were initially based upon pathologic changes associated with each as shown in Table 5. As the clinical picture in these patients unfolded it became apparent that these categories were equally applicable to the clinical and biochemical course of the disorder.[2] Phase I was established as the time between the onset of the illness and the 14th month. Phase II was assigned to the period between the 14th and 24th month of determined illness. Phase III extended beyond 24 months of illness. Sustained clinical observation after 24 months failed to reveal any new features, other than head enlargement, not recorded in the two earlier phases of the illness. This last phase was characterized by progressive inanition which often accentuated the head enlargement. Skeletal muscle atrophy became more prominent as did a generalized flaccidity and a diffuse vegetative immobility.

Serial head circumferences determined during Phase I of the disease showed essentially normal values (usually, however, within the lower limits of normal). Simultaneously determined chest circumferences were also either normal or slightly below

TABLE 5.—*Clinical and Pathological Characteristics of Phases of Infantile Amaurotic Family Idiocy*

Phase I: (0-14 months of illness.)

Clinical: Hyperacusis; loss of ability to perform certain motor acts; progressive weakness; failure to recognize familiar objects; blindness (macular degeneration); retardation of growth.

Pathologic: Gross examination of the brain discloses a moderate atrophy with slight decrease in brain weight, narrowing of cerebral sulci and mild ventricular dilatation. Microscopically, there is widespread neuronal cytoplasmic distention, relatively little ganglion cell loss, minimal gliosis and slight, diffuse, demyelination.

Phase II: (15-24 months of illness.)

Clinical: Child becomes increasingly hypotonic and lapses into a vegetative existence; shows occasional convulsive seizures and episodes of opisthontonus; hyperacusis persists; pupils respond sluggishly to light; mild muscle atrophy present; head circumference mildly increased.

Pathologic: Gross examination of the brain shows normal or mildly increased weight and volume; disproportionate atrophy of cerebellum and brain stem; ventricles of normal volume. Microscopic preparations show a widespread neuronal loss, exhuberant gliosis and diffuse demyelination.

Phase III: (more than 24 months of illness.)

Clinical: Child totally immobile, except for rare periods of convulsion; marked muscle atrophy and generalized emaciation; tendency to decubitus ulceration; almost total hypotonicity and arreflexia; general lack of responsiveness to any form of visual or tactile stimulation; progressive megalencephaly.

Pathologic: Macroscopic examination of the brain shows a significant cerebral expansion (up to 1850 grams) with broadened and flattened surface convolutions; marked atrophy of cerebellum and brain stem; demyelination, extensive edema and cystic degeneration of cerebral white matter; supratentorial ventricular volume decreased. Histologically, there is a protean disintegration of affected neurons and a very severe reactive glial hyperplasia.

normal. Pneumoencephalograms performed under the direction of Dr. A. Lewitan showed mild lateral ventricular dilatation and moderate atrophy of the surrounding cerebral tissues as exemplified by increased amounts of air in the supratentorial subarachnoid space.[1] Retracted and shrunken gyri could occasionally be demonstrated by lateral views. Children dying during this phase of the disease had, at autopsy, a shrunken, mildly gliotic brain. The sulci of the cerebral hemispheres were generally widened. The leptomeninges were on occasion slightly thickened. Brain weights in this phase of the disease were below normal or within normal limits.

During Phase II of the disorder, the head circumferences often increased precipitously (Fig. 1). Occipito-mental circumferences approximately 15% above normal were characteristic of this phase. Contrariwise the chest circumferences continued to diminish in accordance with the diffuse somatic retardation (Fig. 2). Autopsies performed during this period showed a surprising reversal from the trend noted in children sv cumbing earlier in the course of the disorder. The cerebral hemispheres were no longer shrunken but were generally

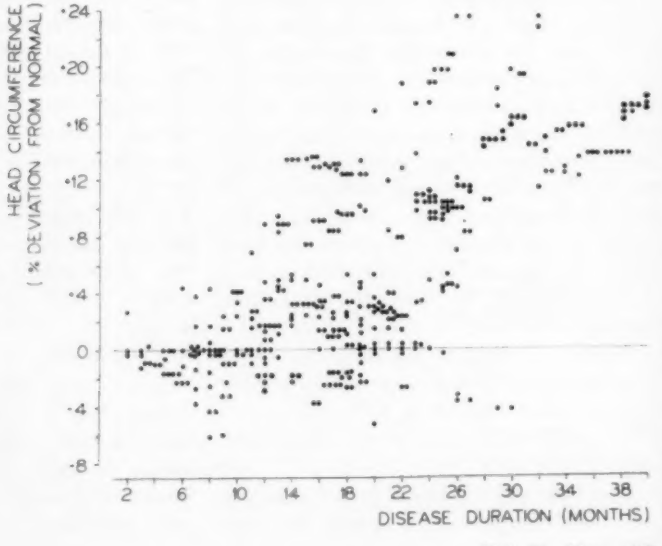

Fig. 1.—Serial head circumference measurements performed on children with amaurotic family idiocy and expressed as percentage deviation from normal.

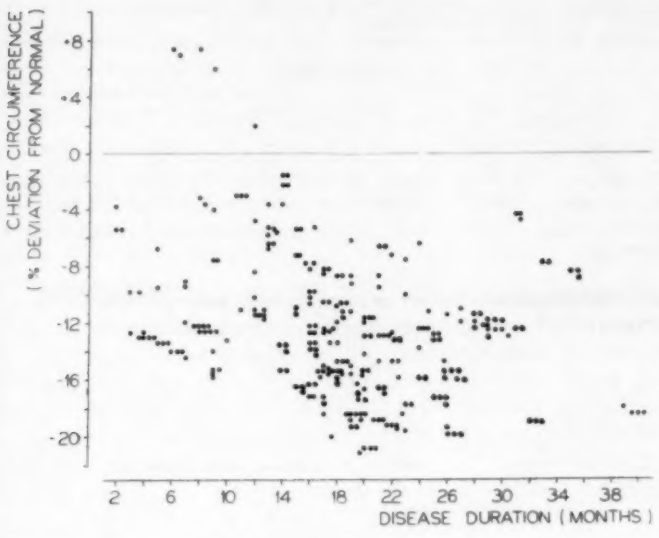

Fig. 2.—Serial chest circumference measurements performed on children with amaurotic family idiocy and expressed as percentage deviation from normal. Compare with Figure 1.

normal or of somewhat increased dimensions. The infratentorial atrophy of the cerebellum, however, continued unabated.

Children surviving into Phase III of the disorder showed progression of the alterations arising during Phase II. Pneumoencephalograms showed a number of changes distinguishing them from the antecedent phases. The bulk of supratentorial tissues were now significantly increased and the lateral ventricles were either of normal dimension or actually compressed. Thus it became apparent that the progressive head enlargement was not attributable to any obstructive hydrocephalus but rather to an actual increase in the bulk of cerebral substance. The chest circumferences of children surviving into Phase III continued to show further retardation.

A compilation of the brain weights obtained at autopsy showed a uniform increase correlated with further duration of the disease (Table 6). Thus, during the first phase of the disease, brain weights tended to be atrophic or within normal ranges. Beyond 30 months of determined disease, however, brain weights averaged over 40% above normal for the particular age and sex. Brain weights of up to 1,850 grams were recorded, normal for this age being approximately 1,160 gm. Specimens from this stage showed cerebral hemispheres that were unusually and uniformly voluminous. There was no evidence of any focal atrophy or abnormal fissuration. In distinct contrast, the cerebellum and the brain stem exhibited atrophy. Serial cross sections through the affected hemispheres showed a uniform pattern in all specimens. The ventricular cavities were either normal or occasionally compressed. The bulk of cerebral tissue was inordinately increased; this increment was derived exclusively from the white matter. The intracerebral white matter was considerably expanded and showed a peculiar form of coalescent, cystic necrosis characterized by ragged areas of encephalomalacia, demyelination and edema. Histologically, the cortical gray matter was the site of diffuse ganglionic involvement. However, in contrast to the previous stages of the illness, most of the neurons had now degenerated completely, leaving whole fields of cortex with no residual ganglion cells but solely a secondary and extensive glial reaction. In apparent response to this generalized supratentorial ganglionic breakdown, the gliosis appeared to be out of proportion to the anticipated response. In many instances the proliferating astrocytes

TABLE 6.—*Development of Megelencephaly in Twenty-three Consecutive Autopsied Cases of Infantile Amaurotic Family Idiocy*

	Age, Months					
	13–18	19–24	25–30	31–36	37–42	43–60
Normal Brain Weight, grams	1045	1055	1080	1120	1160	1190
Brain Weight, A. F. I.*	1065	1195	1445	1632	1710	1718
	(906–1230)	(970–1440)	(1160–1750)	(1500–1830)	(1570–1850)	(1636–1800)
Percentage Deviation from Normal	+1.9%	+13.2%	+33.8%	+45.7%	+47.4%	+44.4%

* Arithmetic mean and, in parentheses, range.

were multinucleated and pseudoneoplastic. In striking contrast, the tissues of the cerebellum showed only a pattern of neuronal ballooning, but no unusual degree of neurocellular necrosis or superimposed glial responsiveness typifying the cerebral changes.

If the body weight of these children is determined at widely separated intervals it is quite apparent that there is a retardation, but not cessation in body growth. If, however, weights are recorded at more frequent intervals, a chaotic and rather bizarre growth pattern becomes apparent. There is a wide weekly variation in body weight which strongly suggests that the integrative control of growth and homeostasis is seriously disturbed (Fig. 3). These grossly fluctuant growth curves have been noted,

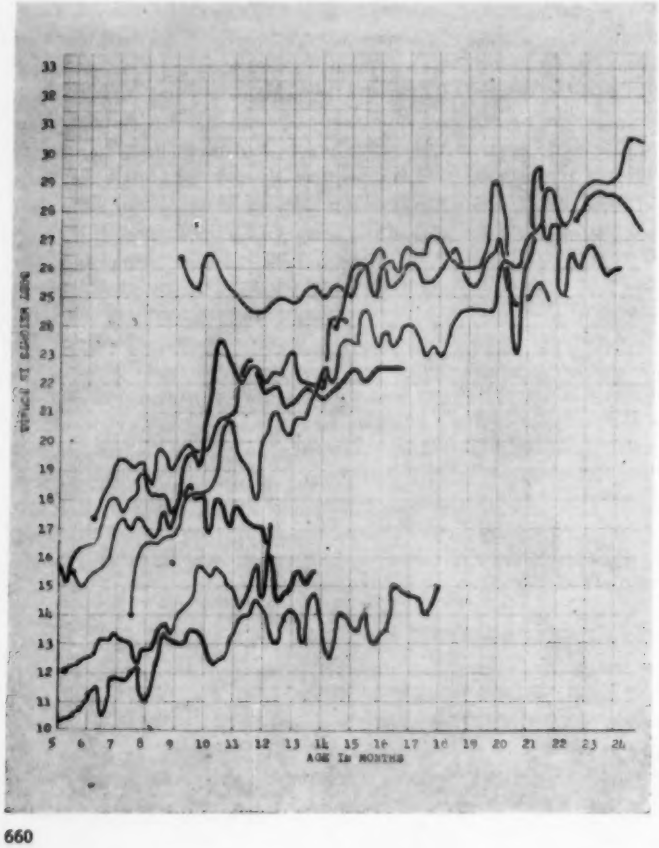

Fig. 3.—Weekly body weights of children with amaurotic family idiocy. Note wide amplitudes.

to a lesser degree, in children with other diffuse neurologic disorders. That this growth pattern is not intrinsic, however, is shown by the fact that when some of the patients were transferred from one of the custodial wards to the general pediatric wards, the latter characterized by more careful climatic and nursing supervision, the wide weekly fluctuations in weight tended to diminish considerably.

It might be germane to mention another finding noted by a small number of the parents of these children and corroborated by the attending physicians. These observers have described an unusual sexual precocity in a small number of the infants. Pubic hair has been recorded in a 30-month-old child with Tay-Sachs' disease.

A determination of bone age was made on seven patients. The readings were performed by Dr. Leo Radwin, using the Todd-Greulich atlas. Of these seven patients, four were considered to have normal bone age; two had moderately retarded bone age and in one, the bone age lagged by about a year.

Comment

When Sachs first described this disease as one of arrested development, his designation was based principally upon the cessation of somatic growth, the loss of functional activity, and the finding of an atrophic brain upon autopsy. Subsequent studies by other investigators have reaffirmed these observations. Most reported clinical descriptions, however, are based upon observations in children who have generally succumbed within 18 months of life.[3]

The brains of the afflicted children became more and more voluminous as the duration of Stage III lengthened. The first phase of the disease may be considered as a stage of progressive intracytoplasmic infiltration with abnormal lipoid material, partial malfunction of involved ganglion cells, retardation of the capacity for further development with mild attendant cerebral atrophy, but no significant breakdown of the altered and diseased nerve cells. When a critical point in the relentless storage process is reached, a diffuse neurocytolysis is precipitated resulting in a structural loss of large numbers of ganglion cells. This crucial change occurs about 18 months after the onset of the disturbance. The ganglionic degradation results in a secondary breakdown of the axonal appendices. A devastating cerebral white matter demyelination develops, often simulating diffuse sclerosis and accompanied by an overwhelming gliosis and edema. The liberated abnormal lipids may very well be the instigating cause of the unusual glial response. As a result of the tremendous gliosis the cerebral hemispheres re-expand, achieving megalencephalic proportions. In reality this represents merely an overwhelming gliogenous hyperplasia. The cerebellum and brain stem however, continue to display an atrophy even in the protracted phases of the disease (Fig. 4).

Histologic examination at varying stages of the disorder have invariably shown a definite hierarchy of involvement. In spite of the ubiquitous intracellular involvement in Tay Sachs' disease, certain areas of the brain appear to be involved more extensively than others. This pattern is not entirely arbitrary but appears to comply with the phylogenetic development of the individual zones of the brain. In general terms the more recently developed areas of

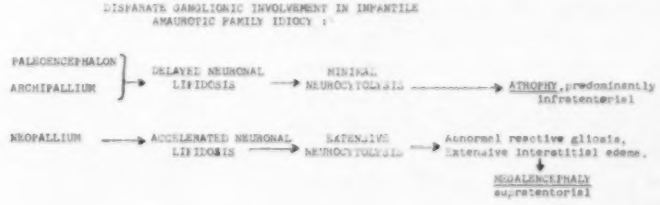

Figure 4

the brain are more prone to an accelerated progression of the disorder while the more archaic areas of the central nervous system generally display a more torpid course. Thus, referring back to the disparity between cerebellum and cerebrum, it is repeatedly noted that the ganglion cells of the former area, while involved, never exhibit the degree of distention or cell destruction characteristic of the cerebral ganglion cell gray matter mantle.

Isaac Albert Research Institute, Jewish Chronic Disease Hospital, Brooklyn.

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Juvenile Amaurotic Idiocy

GEORGE A. JERVIS, M.D.

Juvenile amaurotic idiocy (Vogt-Spielmeyer's disease) was first recognized in 1826 by Stengel,[1] a Norwegian physician. The descriptions of Batten,[2] Vogt,[3] and Spielmeyer[4] some 50 years ago, clearly established the basic clinical and pathological aspects of the disease and, 25 years later, thorough study of a large number of cases which were observed in Sweden[5,6] contributed much to the genetic, clinical and pathological understanding of the disease. In this country, juvenile amaurotic idiocy seems to have attracted much less attention than the infantile type (Tay-Sachs' disease) and only a few reports are available in the medical literature. Yet the disease is not as rare as commonly thought. In fact, from personal observation, the incidence of the juvenile form (9 cases) is not far off from the incidence of the infantile form (15 cases). This ratio of 6:10 was observed in a hospital where there is a high percentage (some 25%) of children of Jewish extraction and therefore a higher expectation of frequency of Tay-Sachs' disease than in other hospitals throughout the United States.

The purpose of this presentation is to review briefly some genetic, clinical and pathological aspects of the disease mainly on the basis of personally observed cases.

1. *Genetic Aspects.*—That the condition is determined by a single recessive autosomal gene was amply demonstrated by Sjögren[5] in a classical monograph which includes exhaustive data on 59 families. This finding needs no re-emphasis. Some of the families personally observed were too small to permit a genetic analysis but in one sibship comprising 15 sibs, six cases of the disease were observed. In examining a sibship, the obvious fact should be kept in mind that sibs below the age of 7 although apparently normal, may still develop the disease. It would be important therefore to uncover some subtle evidence of a developing degeneration of the central nervous system in the absence of gross clinical signs. In this connection, it is interesting to note that the younger sib of one personally observed patient, an intellectually normal child 4 years of age had a definitely abnormal electroencephalographic pattern. A similar observation was reported by Hoffman.[7] Whether abnormalities of electric potential in these children are the first manifestation of the disease will be determined only by future observations.

The ancestry in the cases which were personally observed could be traced to British, Irish, Scotch, German, Italian and African families. Of particular interest is the case, apparently the first recorded, of a Negro girl in whom the clinical diagnosis was confirmed by pathological examination. Cases in Japanese families have been reported.[8] The gene seems therefore to be quite widespread among Caucasian people and present also in African and Asiatic races. In no personal case was there evidence of Semitic ancestry, a fact which is in sharp contrast with the 15 cases personally observed of the infantile form, all of which were in children of unmixed Jewish families. To be sure, the presence of Tay-Sachs' disease in Caucasian families and even in Asiatic populations[9] is well known, but, as far as I know, no Jewish cases of the juvenile type have been reported. This fact, together with other data, is a significant argument in favor of the clear-cut genetic distinction between juvenile and infantile amaurotic idiocy.

From the Research Department, Letchworth Village, New York State Department of Mental Hygiene, Thiells, New York.

In none of the observed cases was there parental consanguinity. The frequency of the gene responsible for juvenile amaurotic idiocy is of the order of 0.0038% in Sweden [5] but no data are available concerning its frequency in the general population of this country. Therefore, it is impossible to estimate the expected frequency of parental consanguinity. However, if this observed low incidence is confirmed by a much larger number of cases, one may infer that the frequency of the gene is not as low as usually considered, since it is well known that the rarer the gene the higher the incidence of parental consanguinity.

2. *Clinical Aspects.*—The clinical manifestations of juvenile amaurotic idiocy and the uniformity of their chronological sequence are too well known to need reemphasis. In all cases observed, mental and physical development were normal during the first 5 years of life and intellectual deterioration began only at the age of 5 to 7 years. The children usually were able to attend the first grade of grammar school but toward the end of it or during the second grade, intellectual failure became manifest. This was accompanied often by incipient visual difficulty. In other cases the first manifestation of the disease was diminution of visual acuity. The close intermingling of mild intellectual and visual failing made it difficult to evaluate the presenting symptomatology at this first stage. With the slowly progressing increase of mental deterioration and decrease of visual acuity the diagnosis became clearer. In all cases, optic atrophy was obvious by the 10th year and retinitis pigmentosa was usually found. Psychotic manifestations developing during the first stage of the disease were noted in four cases suggesting the diagnosis of childhood schizophrenia. It is not impossible that some instances of so-called childhood schizophrenia or of Heller's disease are actually cases of juvenile amaurotic idiocy. Pathological findings of neurolipidosis in cases of "dementia praecocissima" described by Corberi [10] as well as biochemical data reported by Korey [11] in a case of Heller's disease offer some support to this assumption. Fundoscopic lesions are not always found in juvenile amaurotic idiocy as shown by some cases described in the literature [12,13] thus making it difficult to arrive at a correct diagnosis in these hypothetical instances. In all cases personally observed, to be sure, pigmentation of the retina and atrophy of the optic nerve were present and some of the reported instances of Spielmeyer-Vogt's disease, without amaurosis particularly those described by Zierl [12] may have been cases of gargoylism.

Convulsive manifestations were observed in all but one case, by the 10th year of life. In two cases, grand mal attacks were the first serious manifestation of the disease. In the only patient without overt epileptic manifestations, the electroencephalographic tracing was grossly abnormal being consistent with the diagnosis of convulsive disorder. Abnormalities of brain potential have been stressed by Cobb et al.[14] and were confirmed by the study of 3 cases in the present series. In two cases recently described by Jefferson and Rutter [15] overt fits were likewise absent but conspicuous abnormalities of the electroencephalogram were noted.

In the last stage of the disease, various neurological signs were recorded: paralysis in flexion of the extremities, evidence of pyramidal involvement, incoordination of movements, nystagmus, pupillary fixity, etc. In no case was there observed the striking cerebellar symptomatology first described by Frenkel and Dide [16] nor the myoclonic movements which were so characteristic of Liebers' case.[17]

The clinical symptoms and their chronological sequences in the patients personally observed, in summary, conform to the large majority of cases reported in the literature. In all, death occurred between the 14th and 17th year of life.

It should be noted that a small number of patients with marked deviations from this characteristic symptomatology have been reported in the literature, particularly

by Wyburn-Mason.[18] Two such very atypical cases were personally observed but their inclusion in the group of juvenile amaurotic idiocy of the Spielmeyer-Vogt type is doubtful.

Laboratory data in the present series offered nothing of interest: increase of total protein of cerebro-spinal fluid was noted in the majority of the cases. Biochemical abnormalities could not be demonstrated in two cases extensively investigated: urine and blood chromatogram for amino acids, aromatic compounds and sugars were within normal limits. Lipid fractions in the blood studied with conventional methods were likewise normal. Enzyme studies of the blood including phosphatase, transaminase, lactic acid dehydrogenase and aldolase showed no abnormalities. The presence of vacuolated white cells described by Scandinavian investigators[19] could not be confirmed.

3. *Pathological Aspects.*—The highly characteristic pathologic features of juvenile amaurotic idiocy have been thoroughly examined in the past and there is little that can be added to the classical descriptions. In the material personally examined, ubiquitous swelling of the nerve cells and cytoplasmic infiltration with lipid material were outstanding findings. Cellular swelling was however less conspicuous and dendrites were less involved than in the infantile form. Axons showed no swelling with the exception of the axons of Purkinje cells in which localized swelling was not uncommon but there was apparently no lipid material. Cortical nerve cells were diffusely enlarged, giving often the false impression of a cortical field crowded with cellular elements. Destruction of cells was also noted, particularly in the fifth layer. No constant pattern in the intensity of involvement of the various regions of the cortex was observed from case to case. Macroglia showed as a rule, hypertrophic and hyperplastic changes. Infiltration of the glia cytoplasma with lipid material similar in staining properties to that infiltrating the nerve cells was also noted. Oligoglia were inactive. In two cases, microglia were apparently active with formation of numerous hypertrophic elongated forms probably representing a secondary reaction to destructive processes of the nervous parenchyma.

In the white matter, the myelin sheaths were intact in sharp contrast with some cases of the infantile form in which extensive demyelination may be found. Basal ganglia and thalami showed cellular lesions of the same type as those found in the cortex but usually of lesser degree. In two cases, the cerebellum showed "cerebellofugal" type of degeneration characterized by extensive destruction of Purkinje cells and relative sparing of granules. In the spinal cord, the motor cells contained only a small amount of lipid material.

No storage of lipid was found outside the central nervous system.

The *chemical nature* of the lipid material infiltrating nerve cells has been the object of extensive study in the attempt to clarify the nature of the pathologic process and to distinguish juvenile amaurotic idiocy from other forms of lipidosis of the central nervous system. In the case examined, Sudan III and IV stained the lipid pale pink; a similar staining is obtained in other forms of cerebral lipidosis with the exception of cholesterinosis in which the lipid is bright red. With the periodic acid-Schiff method, there is always a striking positive reaction which is however shared by all other forms of amaurotic idiocy suggesting that the lipid contains a carbohydrate residue or a free glycol. It would indicate the presence of gangliosides or cerebrosides. However, this reaction is also positive in a case of Niemann-Pick's disease, a condition in which sphyngomyelin, a substance containing no carbohydrate residue, is presumably the infiltrating lipid. Positive results are also obtained by sulfation and phosphorylation followed by aldehyde fuchsin, indicating the presence of substances which can be converted to acidic groups, probably carbohydrate moieties of lipids or aliphatic hydroxyl groups such as those present in the amino acid serine or thionine. Again these reac-

tions were positive also in other types of amaurotic idiocy. The metachromatic reaction of Feyrter is intensively positive in all tested cases of both infantile and juvenile forms. The Danielli reaction is positive in the two juvenile cases which were tested indicating the presence of amino acids, tyrosine, triptophane and histidine and thus suggesting that the intracellular lipid is bound to a protein. Of special interest was the fact that the Diezel reaction [20] was positive: this staining procedure which utilizes the Bial reagent, presumably demonstrates the presence of neuraminic acid and therefore is said to be specific for ganglioside. Although sections of tissue from patients with Tay-Sachs' disease are intensely positive, the specificity of the reaction is open to doubt. Finally, positive staining is obtained with phosphomolibdic acid-stannous hematoxylin; it has been claimed that with this reaction choline-containing sphyngomyelin can be distinguished from choline-free ganglioside.

Unquestionably, too much emphasis has been placed, recently, on these histochemical tests. As far as differentiation between juvenile and infantile forms of amaurotic idiocy, the tests thus far developed are of little value. The only conclusion that can be derived from histochemical tests is that the lipid infiltrating the nerve cells in juvenile amaurotic idiocy is presumably a sphingolipid, possibly bound to a protein.

The results of chemical examination which was performed using a method of lipid analysis recently developed, showed that a remarkable differential feature exists between juvenile and infantile amaurotic idiocy. While in the latter high values of neuraminic acid and therefore gangliosides were obtained with the Klenk-Langerbeins [21] method, in the former the values were not significantly above the normal limits. It would appear therefore that juvenile amaurotic idiocy is a condition genetically and clinically distinct from infantile amaurotic idiocy. The exact chemical nature of the infiltrating lipid, however, is still undetermined.

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Biochemical Alterations in a Case of Heller's Disease

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In 1909 Heller [1,2,2a] identified a group of mentally retarded children with common symptomatic and historical manifestations. His findings have been confirmed by Zappert, Corberi and others.[3,4] The patients described by Heller were affected between the ages of two to six years in a subacute and progressive manner. Previously normal children manifested signs of impaired conceptualization. This impairment was particularly obvious in the sphere of language. Although the ability to speak words might remain intact, language disintegrated and within a year of the onset of symptoms it was reduced to monoverbal irrelevancies or replaced by sounds and noises. Behavioral changes occurred early. Psychomotor agitation, occasionally associated with apparent hallucinatory episodes, was followed by apathy or a variably excited state of idiocy. In several instances convulsions appeared both prior to the clinical recognition of the syndrome as well as during its course. However epilepsy has not been considered an essential or integral component of the symptoms. The facies of the patients remained somewhat expressive and not without comprehension. Neurological examination showed minor or inconstant deficits. There was no known genetic predisposition. The symptoms were profound within a year of their onset and the prognosis hopeless. Despite careful clinical consideration, the complex of symptoms found by Heller can be confused with those that may occur in autism, amaurotic family idiocy, diffuse sclerosis, as well as in other regressive disorders of the developing nervous system.

The pathological data,[4] though sparse to date, suggest that the syndrome is organically determined. There is a diffuse involvement of cortical cells, Purkinje cells and the larger cells of the brain stem. There appear to be major changes in the lipid content of these cells with evidence of accumulation of some form of lipid in their cytoplasm. The nuclei of these cells become pyknotic and displaced to the periphery of the cell by the increasing amount of intracellular lipid. The staining characteristics of this material are not yet well defined. Degenerated neural cells are abundant. Glial cells appear hypertrophic and hyperplastic. Fat from disrupted nerve cells becomes phagocytized and reaches the perivenous areas and venous channels in appreciable amounts. The histopathological features are similar to those of amaurotic family idiocy but there is no report that establishes the identity of these two syndromes, nor have the retinal changes of Tay-Sachs' disease been noted in cases of dementia infantilis.

Report of Case, June, 1955

The patient is a 5-year-old white female who was well until the age of two and one-half years. The parents date the onset of illness to the family's removal to a new home at that time.

The initial symptoms involved language and behavior. The expected increase in the vocabulary of the patient did not evolve. Rather there was a loss of words and phrases which progressed steadily until the age of three years and three months when there appeared to be an acceleration in the

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impairment of speech. Articulation became gross and sounds were substituted for language. There was an inappropriate use of the few words remaining to the child. A gradual diminution in range of interests and execution of play acts also was noted. Stories which formerly held fascination for the patient were ignored. The patient sat listlessly turning pages of a book unmindful of whether or not it was correctly oriented. With the apparent increase in tempo of symptoms, the child became utterly dependent on the mother, refusing to leave her side at all. Toilet habits deteriorated. The advent of a second sibling augmented the patient's abnormal behavior. Several times she unexpectedly assaulted the infant.

The mother stated that the patient was born without difficulty after a normal pregnancy. Birth weight was 3.97 kg. The patient breathed and cried spontaneously at birth and appeared to develop from this time apparently normally. The patient held her head up at three to four months, rolled over at six months, sat up without support at six months, stood at 11 months and walked alone at 13 to 14 months. Toilet training, which was started at 14 months, was completed at 29 months. At the age of 12 months, the patient spoke words, recognized strangers and explored her surroundings with interest. Her language abilities progressed, although sentences were never formed and spoken with clarity. She obeyed simple commands by the age of 18 months and dressed herself with some assistance shortly thereafter. She knew her name and listened attentively to stories read to her by her parents. She played simple games with her brother and occupied herself by towering blocks. There were no sleep disturbances.

The child was quite healthy except for measles at the age of three years and seven months, varicella at 5 years, and occasional upper respiratory infections. The infectious diseases were self-limited.

The mother, American born, is 33 years old, a healthy housewife who has had five pregnancies, one of which terminated in a spontaneous abortion at the 10-week period. The father, Finnish born, is a diligent worker who is entirely well. Both parents are generous, comprehending and affectionate. The three siblings of the patient, aged 6 years, 15 months, and one month, are active and apparently healthy. With the exception of a cousin of the patient who had idiopathic epilepsy, there is no history of nervous or mental disorders in the family.

The patient was admitted to the Babies' and Children's Division of the University Hospitals upon recommendation of Dr. Jane Kessler, the examining psychologist, who suggested the diagnosis of Heller's disease.

Physical examination revealed a well-developed, well-nourished, white female child weighing 18.4 kg. She had a somewhat troubled expression which was neither vacuous nor demented and might be taken as reasonably normal for an anxious child. She was entirely uncooperative and all examinations and procedures were performed under duress. The general physical examination was normal. Neurological examination was unusual in only one respect—a Babinski sign was found on occasion on the left. Psychological testing was impossible. We were limited to observing the patient's activity on the ward and in contrived play situations which had no appeal to her. The psychiatric and pediatric staff concluded that the patient had an organic dementia.

The patient wandered about the hospital ward aimlessly, occasionally stopping to glance at the shiny door knobs. She appeared oblivious to the attendants and other children and formed no personal attachments. Picture books, games and objects were fingered but not investigated. There was virtually no communication established with the patient. She expressed inconsolable terror by cries and bodily activity whenever a routine venipuncture was attempted. There were no destructive acts. Stereotyped hand movements were a marked feature. The patient placed the dorsal aspects of her hands against her eyes and bent her fingers downwards. She appeared to sniff at them. Frequently her fingers were stiffened and adducted with her hands at rest and with no apparent associated bodily tension. For the most part her mood was estimated to be vaguely apprehensive, but this was uncertain. She ignored bed restraints and did not respond to direct confrontation. Perhaps the examinations and diagnostic procedures which induced howling and crying left her with a persistent feeling of anxiety. She had no sleep disturbances.

Although her motor power and coordination were unaffected in simple acts she was unable to feed herself with utensils. She stuffed food into her mouth with her fingers. At home she was frequently seen to eat raw meat taken from the refrigerator. She may have had pica prior to hospitalization. On the ward her appetite was not remarkable, but periodically before entry into the hospital and subsequent to discharge it rose to great heights, and food, especially meat, was ravenously consumed. The patient was unaware of her excretions and indifferent to any hygienic measures.

Response to questioning or commands was not observed. Spontaneous noises, grunts, and occasional screams and shrieks were uttered. Sometimes catch phrases of television commercials were repeated directly after they were heard, but no sustained language existed.

The patient recognized no one but her mother and even with her, personal recognition was not regular and was extremely superficial. The patient was indifferent to her mother's departure from the ward, but voluntarily sat beside her when she returned.

The parents' attitude was reasonable. They were certain that the child was normal prior to the present illness. They could not fully understand the present course of events. They were anxious to keep the child at home if any possible assistance could be offered, but recognized the probable need for institutionalization.

Laboratory Studies.—The results of the general laboratory examination were as follows: Hemoglobin 14.5 grams; RBC 4.8 million/mm.[3]; Hematocrit 43%; WBC 13,200 and an 9,250/mm.[3]; no basophilia was noted. The differential count was normal. Routine analysis of the urine was negative. Total urinary protein was 146 mgm. %. Neither coproporphyrin in increased amounts nor phenylpyruvic acid was detected in the urine. Excretion of lead in the urine was 0.07 and 0.31 mgs./l. in separate determinations. The normal level for urinary excretion was considered to be 0.08 mgs/l. Excretion of copper was 31.75 micrograms/500 ml. of urine. Examination of the serum yielded the following values: urea nitrogen 12.7 mg. %; fasting sugar 79.5 mg. %; total protein 7.1 gms. %; albumin 5.25 gm. %; globulin 1.85 gm. %; A:G ratio 2.8; sodium 148 m.eq./l; potassium 4.4 m.eq./l and chlorides 109.9 m.eq./l. Serum calcium was 10.84 mg./% and inorganic phosphorus, 4.67 and 4.78 mgs. %. The values of the alkaline phosphatase corresponding temporally to the determinations of serum phosphorus were 2.53 and 2.93, 3.38, 2.16 B.U. The cephalin flocculation test was 2+ on one examination and 0 on others, thymol turbidity 0.9 units, serum bilirubin 0.4 mg. %, prothrombin 100%. Iodine ^{131} uptake in 24 hours was 13%, protein-bound iodine was 4.53 micrograms % and 6.2 micrograms %. Serum lead was 0.2 mgs. per 100 grams of blood. Cold agglutinins were negative. The PPD in 1-100 dilution was negative. Radiographic examination of the chest was negative. No evidence of heavy metal deposition in the long bones was found. The bone age corresponded to the chronological age. The electroencephalogram was normal both during waking and sleeping states. The results of the cerebrospinal fluid examination were: initial pressure 210 mm. of water, final pressure 150 mm. of water; 10 cc. of spinal fluid were removed. The cerebrospinal fluid protein was 21.3 and 23.5 mgs. %; chlorides 124.5 m.eq. %; sugar 72 mgs. %. Pneumo-encephalogram performed via the lumbar route was entirely normal. The blood and spinal fluid serological examinations for syphilis were negative.

Lipid Partition.—The lipids of plasma and cerebrospinal fluid were extracted in similar fashion. However, due to the difficulties in dealing with large volumes and the consequent inefficiency of the extraction procedure urine was lyophilized first. The dried residue was resuspended in a small amount of water and then extracted as indicated. Twenty volumes of $CHCl_3$-CH_3OH, 2:1 (v/v) were blendorized with one volume of the fluid for 3-5 minutes.[6] The suspension was filtered. The lipid extract was dialyzed against several changes of distilled water for several days. The watery layer of the dialysate was separated from the underlying chloroform layer. Each layer was concentrated to dryness and redissolved in chloroform: methanol and rapidly refiltered. Lipid weights were obtained by evaporating an aliquot of this extract to dryness on a steam bath under a gentle stream of air. The weighing vessel was then heated in vacuo overnight at 65° and then allowed to cool at room temperature. An aliquot of lipid extract was evaporated to dryness and its nitrogen content determined by the Kjeldahl procedure. Lipid phosphorus was determined following perchloric acid digestion of a previously dried aliquot of the lipid extract.[6] References to the other determinations follow: total fatty acids and fatty acid unsaturation[7]; plasmalogen[6]; cholesterol[8]; "cerebrosides"[9]; α amino nitrogen[10]; neuraminic acid[11]; red blood cell cerebrosides[12]; serum glucosamine.[13] In order to detect the presence of a lipoprotein similar to that found in spleens of patients with Gaucher's disease both the plasma and red blood cells of this patient were examined by the procedure described by Uzman.[14]

Electrophoretic Studies.—Plasma was studied in a moving boundary Tiselius apparatus in 0.1 ionic strength sodium diethyl barbiturate buffer, pH 8.6, and by means of paper electrophoresis.[15] In the latter method the plasma components were stained by bromthymol blue, oil red, periodic acid-Schiff and sudan black. Hemoglobin likewise was electrophoresed on paper.

Urine was chromatographed on Whatman #1 paper suitable for chromatography in a 2-dimensional system. Phenol water 4:1 (V/V) and collidine: lutidine: water (1:1:1) were the solvents.[16] Authentic samples of amino acids were carried through with each determination.

Alpha amino nitrogen present in the urine was determined by the method of Hamilton et al.[10]

Attempts to separate chromatographically the sugars liberated by hydrolysis of the plasma cerebrosides were unsuccessful due to a persisting contaminant which changed the partition characteristics of the sugars sufficiently to render the distinction between galactose and glucose uncertain. However amino hexoses could be distinguished as could sugars of different chain length.

TABLE 1.—*The Plasma Lipid Profile in a Case of Dementia Infantilis*

Determination	Amount
L. W.	1112 mgm. %
L. N.	1007 μM. %
L. P.	281 μM. %
LN / LP	3.58
Phospholipids *	225 mgm. %
Plasmalogen	27.7 μM. %
Plasmal-P / L. P.	0.10
Plasmal-P / L. W.	0.019
Total fatty acids	871 μM. %
Iodine	2543 μM. %
Iodine No.	58
Cholesterol	167 mgm. %
Esters	103.5 mgm. %
Ester / Total	0.62
"Cerebrosides"	30.5 mgm. %
L. αNH_2N	89.1 μM. %
L. αNH_2N / L. P.	0.32
Neuraminic acid	55.5 mgm. %

L. W., lipid weight; L. N., lipid nitrogen; L. P., lipid phosphorus; L. αNH_2N, lipid alpha amino nitrogen; Plasmal-P, the phosphorus found in the plasmalogen fraction.

* Phospholipids were calculated by multiplying L. P. × 25.

TABLE 2.—*The Analysis of Several Body Fluids and Red Blood Cells for Particular Components*

Source	Determination	Amount
Plasma Lipid Supernatant		
	L. N.	64.5 μM. %
	L. P.	3.1 μM. %
	LN / LP	20.8
Serum	αNH_2N	5.4 mgm. %
	Glucosamine	80 mgm. %
Red Blood Cells		
	Cerebrosides	0.1%/gm. dried cells
Cerebrospinal Fluid		
	Protein	23 mgm. %
	L. W.	11.9 mgm. %
	L. N.	29.1 μM. %
	L. P.	1.6 μM. %
	LN / LP	18.2
Urine		
	L. W.	31.2 mgm. %
	αNH_2N	1.88 μM/100 ml.
	Amino acids	Phosphoserine g. amino butyric acid

There were in addition to the two noted in the urine a variety of normally occurring amino acids. The abbreviations are the same as in Table 1.

Results.—The results of the various analyses of plasma, cerebrospinal fluid, red blood cells and urine are presented in Tables 1 and 2. Electrophoresis of the plasma proteins and examination of the hemoglobin by paper electrophoresis revealed no abnormalities. Moreover the plasma and red blood cells were investigated with negative results for the abnormal lipoprotein described by Uzman[14] in a case of Gaucher's disease. The plasma lipid profile is of interest with regard to the lipid weight and the unusual LN/LP ratio. The lipid weight ranged from 1,100 to 1,300 mgm. % about twice the amount normally present. The lipid nitrogen was extremely elevated. As a consequence the LN/LP ratio was high. The examination of sera from 12 unclassified mentally defective children as well as normal children and adults has revealed a range of lipid nitrogen from 266 to 500μM. %.* In addition the radio, LN/LP, is usually within the limits of 1.05 and 1.6. It is important to note that for this analysis the serum must be entirely free of hemolysis and its lipid extract extensively dialyzed. A lipid contaminant that makes its appearance in the course of hemolysis has a significant content of nitrogen. The need for prolonged dialysis has been stressed previously[17] since urea and non-protein nitrogenous products may affect the lipid nitrogen determinations. Although the plasmalogen content of the plasma was believed to be increased when this case was presented originally[18] more extensive study of the sera of children indicates that the plasmalogen content in children may be as high as 30μM. %. Several examinations of the plasmalogens in the plasma of this patient revealed a relatively uniform concentration of 25-28 μM. % which constituted about 8% of the total phosphatides by weight. The presence of 30 mgm. % cerebrosides by the method employed[9] is a borderline finding since in children there usually is a trace to 15 mgm. % cerebrosides or about 2% of the neutral fat content in plasma.[19,20] The amount of neutral fat present can be approximately estimated since the lipid phosphorus is known and the molar ratio of fatty acid/LP is 2:1. The remaining fatty acid may be attributed to neutral fat to furnish an upper value of about 80-90 mgm. %. There is no accumulation of this compound. The lipid α NH_2-N and its ratio to total LP as well as the content of neuraminic acid in plasma are normal.

* Dr. George A. Jervis graciously supplied plasma samples from mentally deficient patients hospitalized in Letchworth Village. Although it was hoped that these patients might be examples of Heller's disease none of their histories corresponded to the general description of dementia infantilis. The analyses of the content and distribution of lipid in their plasmas served as controls.

On the basis of the various determinations of the lipids in the plasma only about 500-600 mgm. of the total 1,100 mgm./100 ml. can be accounted for. The possibility that a lipoprotein such as the proteolipids normally absent from plasma could have been extracted into the $CHCl_3$ methanol solutions was not excluded in the first examination (v. i.). Thus the disparity between the calculated lipid weight and that experimentally found as well as the elevated lipid nitrogen content in plasma are unexplained. The significance of the amounts of lipid in the supernatant of the lipid extract of plasma cannot be assessed since there is insufficient data for comparison but the values are included here for purposes of future reference. The content of lipid in the cerebrospinal fluid is greater than occurs normally.[23,23a] Moreover the lipids are disproportionately increased with regard to total proteins. There is little information concerning the changes in lipids in the cerebrospinal fluid in pathological states but variations have been noted in multiple sclerosis, degenerative syndromes, and neoplasms.[23] The elevated LN/LP ratio likewise is unusual for cerebrospinal fluid[23a] and is suggestive that a compound similar to that present in the plasma may exist in the cerebrospinal fluid.

The urinary findings in Table 2 deserve comment in that the output of lipid apparently is increased to a pathological degree.[23,32] However the procedure employed for extracting lipid from urine is not usual and the range of the urinary lipid content at different ages as obtained by this method is not available at this time. Of the amino acids detected phosphoserine and gamma amino butyric appeared of interest. Phosphoserine was present consistently. Gamma amino butyric acid was found occasionally and not in amounts comparable to other acids. The urinary αNH_2-N was considered to be within normal limits. Abnormal values for urinary and serum leads were obtained a month after the first normal determinations were made and well beyond the full blown development of the patient's syndrome. The values obtained were regarded as suspect since there were no associated findings of plumbism noted in the clinical and other laboratory examinations.

Due to the kind assistance of Dr. Judith H. Rettig, Clinical Director of the Columbus State School, where the patient is hospitalized at present (1958), we have been able to examine some of the critical constituents of the body fluids described 4 years previously (Table 3). The lipid weight of the plasma has reverted to normal but the distribution of some of the other lipid components appears unusual. The lipid phosphorus is depressed. The ratio of lipid nitrogen to lipid phosphorus is excessively elevated. The large amount of lipid

TABLE 3.—*The Determination in 1958 of Some Components of Plasma, Cerebrospinal Fluid, and Urine in the Case of Heller's Disease Originally Investigated in 1954*

	Amount	Ratio
Plasma		
Lipids		
L. W.	440 mgm. %	
L. N.	56 μM. %	
partition		
water soluble		0.21
$CHCl_3$ soluble		0.79
L. P.	13.2 μM. %	
LN/LP		4.3
L. O. acyl ester	542 μM. %	
Cholesterol (total)	87.5 mgm. %	
L. "sugars"	88.6 μM. %	
Lipid supernatant	no material	
Serum		
Alkaline phosphatase	11.2, 10.8, 11.3 B. U.	
Neuraminic acid total	76.8 mgm. %	
globulin neuraminic acid	40.1	
GN/TN		0.52
Cerebrospinal Fluid		
Total protein	22 mgm. %	
Total neuraminic acid	4.1 mgm. %	
Bound neuraminic acid	0.63 mgm. %	
Urine		
L. W.	10-14 mgm. %	

The abbreviations are similar to those of Table 1 with the following additions: L. O. acyl ester—the O acyl esters of the lipid extract (28); L. "sugars"—the substances reacting with orcinol (24); GN/TN—the ratio of globulin bound neuraminic to total neuraminic acid. Partition refers to the distribution of nitrogen between aqueous and non-aqueous solvents subsequent to hydrolysis of the lipid extract.

sugars[24] cannot be attributed solely to cerebrosides since lipid nitrogen is not in an equivalent range. To examine the partition of lipid nitrogen the lipid extract was hydrolyzed at 95° for 5 hours in 5N HCl and for 1 hour in 12N HCl and then thoroughly desiccated to remove HCl acid. The residue was suspended in water and extracted with $CHCl_3$. Both layers were re-extracted by the companion solvent several times. The nitrogen extractable by water was about 21% of the total nitrogen of the serum lipid nitrogen and corresponded closely to the αNH_2-N determined in the aqueous extract.[28] The remaining 79% of the total nitrogen was found in the chloroform extract and was presumably lipid nitrogen, e. g. sphingosine. The amount of αNH_2-N found corresponded closely to the lipid P present. This would appear to eliminate proteolipids as a source of the relatively increased value for nitrogen in this examination. The aqueous supernatant of the lipid extract of plasma was lyophilized but no material was noted in contrast to the previous experience. Of unusual interest is the diminished amount of globulin-bound neuraminic acid found in the cerebrospinal fluid. This decreased content of bound neuraminic acid

is found in instances of Tay-Sachs' disease.† The serum neuraminic acid showed no deviation either in total amount or distribution. Serum alkaline phosphatase measured again by the Bodansky procedure was normal. Urinary lipid content as extracted from the lyophilized residue of urine has remained elevated although to a lesser extent. The urinary lipid sample when dried was oily and brownish in appearance. Although dried by many means to a persistent constant weight the sample was waxy and the lipid weight cannot be regarded as precise but rather within the range indicated.

The plasma lipids were chromatographed on silicic acid impregnated paper and developed with 16% methanol in chloroform.[28] Staining with Rhodamine B indicated that the major component of the lipids was in the region of the neutral lipids. Staining with the Schiff reagent accentuated the forward zone of neutral lipids and likewise demonstrated two additional faint spots corresponding to the regions of plasmalogen and phosphatidyl ethanolamine. The glycolipids which migrate close to the neutral lipids would be masked considerably in the present system.

According to the present clinical examinations the status of the patient is unchanged.

Comment

The clinical characteristics mentioned by Heller[1,2] and Zappert[3] with regard to dementia infantilis apply to the present case —namely: normal physical and mental development in the first years of life; onset of the illness in the third or fourth year; regressive changes in the spheres of language and behavior, in particular a diminished attentiveness and responsiveness; preservation of motor functions; paucity or absence of focal neurological signs; retention of an intelligent expression despite progressive dementia. Although such a grouping of signs is distinctive there is no assurance that it is unique. Associated biochemical features might add to the differentiation of this uncertain and rare syndrome.

Of the several examinations performed in this case, the pattern that emerged in 1954 was one of an increased content of lipid of undetermined nature and origin in the blood, cerebrospinal fluid and urine, an elevated serum LN/LP ratio, a questionable increase in "cerebroside" level and a persistently depressed serum alkaline phosphatase in the face of normal physical development. These findings are not present in any of the lipoidoses[20] of which Heller's syndrome may be an example. Although in the cases of Gaucher's disease reported by Bloem et al.[27] the LN/LP ratio in the plasma was increased, the techniques used for lipid extraction by these authors assure contamination by urea[17] and thus a falsely high LN. The lowering of the serum alkaline phosphatase may be interpreted as a manifestation that this syndrome includes systems in addition to the central nervous system and perhaps processes other than those dealing directly with lipid metabolism.

The source of the excess lipid in the blood of this patient is unknown. It is possible that part of it may be derived from the central nervous system. The histopathology of Heller's disease does demonstrate that there is a migration of lipid laden phagocytes to venous channels where they are seen to invade these vessels prior to entering the general circulation.[4] However, without further information about the turnover rates involved as well as the identity of the lipids present in the blood and the urine the answer cannot be approached.

An elevation in urinary lipid content has not been recorded in nephrosis[23] or in the lipoidoses.[20] It adds to the complexity of the picture. Other data do not provide any indication whether the lipids arise from the kidney itself or are transported there for excretion. Since the kidney is able to prevent excretion of many plasma lipids which may be in excess it is possible that the lipids

† We are grateful to Mr. A. Saifer of the Isaac Albert Research Institute for examining the serum and cerebrospinal fluid with respect to their content of neuraminic acid by methods developed in his laboratory. The range of values he has reported are:

Serum total neuraminic acid	77.4	mgm. %	±7.3
Serum globulin neuraminic acid	42.3	mgm. %	±4.6
GN/TN ratio	0.54		±4.3
Cerebrospinal fluid total neuraminic acid	5.1	mgm. %	±0.3
Protein bound neuraminic acid	1.2	mgm. %	±0.07
Free neuraminic acid	4.0	mgm. %	±0.3

cleared are not native to plasma, that an impaired renal mechanism is responsible for the lipuria or that the method used to extract lipids from urine is more effective.

The additional information gathered from the recent (1958) investigation of the patient suggests that the alterations noted in the initial study may represent those associated with the early developing and more intense phase of the illness. This would apply particularly to the increased amount of plasma lipids especially if they were derived originally from breakdown of neural tissue. Nevertheless there are persistent abnormalities found in the present study that might indicate a continuing though less active process. The LN/LP ratio is 4.3. Eighty per cent of the total lipid nitrogen following hydrolysis remains chloroform soluble. There is an excess in lipid "sugars" with regard to other lipid referents. In view of these findings it is suggested that a fragment of a lipid compound such as a ganglioside may be present in the general circulation. Indeed such an inference may be correlated with the decrease in the bound neuraminic acid in cerebrospinal fluid to levels seen in cases of amaurotic family idiocy and to the postulated similarity of the pathological processes in these two syndromes. In this case Heller's disease can be considered a variant of amaurotic family idiocy in which there may be formation of an unusual lipid compound by peripheral tissues or escape of lipid from the brain by cellular transport or through a damaged blood brain barrier.

Attention should be called to the unequivocal reversal of the serum alkaline phosphatase which has risen to normal levels as the disease appears to be assuming a state of relative equilibrium clinically and chemically. Hypotheses with regard to the relation of phosphatases to lipoidoses have been advanced by Sobotka (this symposium) but there is considerable uncertainty about the mechanisms proposed.

Summary

The present report summarizes the investigation of a patient whose illness has the characteristics ascribed to Heller's syndrome. The original examination revealed the presence of an organic dementia and chemical changes mainly in the lipid series of the body fluids and in the serum alkaline phosphatase. Re-examination four years later demonstrated that no significant change in the clinical status of the patient had occurred. There was, however, some modification in the chemical pattern of the disease in that there was reversal of the plasma lipid weight to normal but a preservation of an unusual LN/LP ratio, an elevated amount of lipid "sugars" and a possible lipuria. Moreover, the level of cerebrospinal fluid bound neuraminic acid was similar to that in Tay-Sachs' disease. It is suggested that Heller's disease is a variant of Tay-Sachs' disease and that part of the chemical changes in the former syndrome may be due to the distribution outside the brain of a component or fraction derived from the gangliosides.

We are indebted to Miss M. Orchen and Miss J. Orloff for their assistance in this study.

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Cerebrospinal Fluid, Serum, and Blood Investigations in Amaurotic Family Idiocy

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Laboratory studies, to be described presently, were performed on cerebrospinal fluid and blood of patients with infantile and juvenile amaurotic family idiocy.

Lipase Studies on Cerebrospinal Fluid

Much care was taken to improve Cherry and Crandell's method of lipase determination [22] through purification of the olive oil, use of penicillin to prevent bacterial growth and standardization by means of comparison with the effects of commercial lipases. Table 1 summarizes our results indicating that the lipase content in Tay-Sachs' disease is essentially the same as in a number of other neurological disorders. It is evident that further studies will have to be directed to other enzyme systems in an attempt to detect some of the basic biochemical disturbances underlying this disease.

TABLE 1.—*Lipase in Cerebrospinal Fluid of Children with Neurologic Diseases*

Diagnosis	No. Cases	Lipase Values	Lipolytic Activity Expressed in Mm. of Fatty Acids per 100 c. c. CSF *
Tay-Sachs' Disease	4	0.06	0.3
Spielmeyer-Vogt's Disease	1	0	0
Meningitis	9	0.07	0.35
Hydrocephalus	12 (27 samples)	0.075	0.38
Convulsions	17	0.050	0.25
Mental Retardation	4	0.007	0.035
Optic Atrophy	3	0.42	2.1
Blindness due to involvement of the CNS	3	0.54	2.7

* Computed from the lipase values.

Serum Protein Studies

Paper electrophoresis for protein fractionation of the serum has been used by Saifer, Aronson, Zymaris and Volk in 7 cases of Tay-Sachs' disease.[21] Our comparable studies [23] based on sera from 8 patients,* together with specimens derived from cases of juvenile amaurotic family idiocy and sera from normal controls are summarized in Table 2. Bromphenol blue was used as the staining reagent and a Photovolt No. 525 instrument for recording. The records were drawn on graph paper permitting planimetric evaluation. It would seem to be of interest that there is a difference between the albumin-globulin ratio found in children with Tay-Sachs' disease and in normal children. The t value of 3.8 indicates that the difference is significant at the 5% level. It is improbable that this difference is specific for this type of disease; it may be caused by the concurrent cachexia or by the metabolic effects of pathologic changes in centers of the autonomic system (e. g., hypothalamus) as described by Lomax.[14]

The results of pherograms of the lipoproteins in 7 cases of amaurotic family idiocy and controls are summarized in Table 3. The method applied is that described by Goldbloom, Boyd and Stern [9] using Oil Red-O.

It was possible to distinguish the lipid accumulation in 2 regions of the spectrum: the alpha$_1$ and alpha$_2$ globulin regions, and

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* We are thankful to Dr. S. M. Aronson for kindly providing some of the material.

TABLE 2.—*Serum Proteins in Infantile and Juvenile Amaurotic Family Idiocy*

Diagnosis	No. Cases	Albumin %	Globulin				Alb/Glob.
			α_1 (%)	α_2 (%)	β (%)	γ (%)	
Tay-Sachs' Disease	5	50.6±2.04	3.9±0.7	11.9±2.57	13.1±2.54	20.5±3.07	1.03±0.09
Tay-Sachs' Disease *	1	42.7	4.8	15.8	15.1	21.6	0.90
Spielmeyer-Vogt's Disease	2	47.7±3.7	4.0±0	9.7±0.3	14.4±0.3	22.8±1.2	0.91±0.14
Normal Children	5	54.1±2.13	4.2±0.13	11.1±2.07	15±1.47	15.6±3.09	1.22±0.06
Normal adults	3	47.8±4.31	3.7±0.22	10±1.51	12.8±2.9	25.7±4.47	0.92±0.16

* The serum of this case had been kept under refrigeration for 1 month before electrophoresis.

the beta and gamma globulin regions. No significant differences seem to exist between the lipoprotein content of the serum of normal children and from those with Tay-Sachs' disease.

Hematologic Studies

Up to the present time, vacuolized lymphocytes (v. l.) in peripheral blood have been described only in the juvenile form of amaurotic family idiocy (Spielmeyer-Vogt type). Bagh and Hortling in 1948 [2] were the first to record such changes. Their study of 6 cases indicated that the percentage of v. l. is dependent upon the duration of the disease. Rayner [20] in addition, found v. l. in apparently normal parents and siblings of such patients and explored this occurrence from the point of view of heredity. Nissen [18] and Gilje and Nissen [8] found v. l. in 6 of their 14 patients and believed that the presence of v. l. permits an early diagnosis. Recently Stubbe-Teglbjaerg and Plum in 1955 [24] found v. l. in 11 cases of Spielmeyer-Vogt's disease, and Juliao, Canelas and Longo [12] described 3 analogous cases in Brazil, two of the three showing v. l. This is of certain interest, since Hoffman [10] claims the existence of a "non-Scandinavian" strain in which v. l. are not demonstrable. No findings of this nature in Tay-Sachs' disease are mentioned in the available literature. Kozinn et al.[13] state that no change in the peripheral blood of Tay-Sachs' disease have been thus far observed. In a more recent review, Friedrich, in 1957 [7] mentioned the occurrence of vacuolization in the lymphocytes only in association with the juvenile form of the disease. Vacuolated lymphocytes and monocytes have also been described by Blackfan, Diamond and Leister [5] in Niemann-Pick's disease. Recently these findings have been confirmed by Crocker and Farber [5a] in 18 similar patients but not, however, in the parents or siblings of the afflicted children.

Before discussing our hematologic results in certain lipoidoses we want to summarize our own observations and those of other authors in disease states other than the lipoidoses. Vacuolized cells are found in lymphatic leukemia, necrosis of the pancreas (Naegeli),[13] infectious mononucleosis (Wintrobe),[25] and chronic bacteremia (Blackfan).[5] We were able to confirm these findings in 2 cases of lymphatic leukemia. We were unable to detect v. l. in 9 out of 10 so-called normal children. One child (8 years old) did show one vacuolized lympho-

TABLE 3.—*Serum Lipoproteins in Infantile and Juvenile Amaurotic Family Idiocy*

Diagnosis	No. Cases	Globulin			
		α_1 (%)	α_2 (%)	β (%)	γ (%)
Tay-Sachs' disease	5	26.7±1.70	5.2±0.82	43.2±3.05	20.9±2.26
Tay-Sachs' disease *	1	24.5	6.	41.5	28.3
Spielmeyer-Vogt's disease	1	29.5	4.5	40	26.0
Normal children	5	27±4.21	5.6±1.12	42.3±4.74	25.1±3.52
Normal adults	3	23.5±3.01	4.7±1.0	46.0±5.24	25.8±4.12

* The serum of this case had been kept under refrigeration for 1 month before analysis.

TABLE 4.—*Hematologic Studies in Tay-Sachs' Disease*

Case No.	Sex	Specimen No.	Vacuolated Lymphocytes (%)		
			Patient	Parents	Siblings
I	male	1	5%	Father-2-3%	----------
		2	13%	Mother-10%	----------
II	male	1	5%	Father-5%	----------
		2	8%		
III	female	1	4%	----------	----------
IV	male	(died 8 years ago)	----	Father-30%	----------
				Mother-50%	
V	male	(died 3 years ago)	----	Father-10%	brother-12%
				Mother-20%	
Va	male	(died 4 years ago)	----	Mother-4%	----------

cyte per 150 white cells (3 in 450); this child was in the hospital for tonsillectomy. Wintrobe mentions that the pharyngeal angina of infectious mononucleosis is often confused with tonsillitis. The v. l. disappeared upon recovery after 3 weeks.

The hematologic findings have been summarized in Table 4.

The patients I-III have been examined at St. Christopher's Hospital. The clinical diagnosis of infantile amaurotic family idiocy (Tay-Sachs' disease) was based on the onset of clinical symptoms within the first 3-6 months which eventuated in the clinical triad of paralysis, dementia and amaurosis associated with the typical cherry red spot in the fovea centralis. These children are still alive at the time of this presentation. Blood smears were made of 2 patients at various intervals, in 1 patient only 1 series of smears was available. Routine May-Greenwald and Giemsa stains were employed. A Zeiss microscope with magnification up to 1800 × was used in the study of the slides, and at least 300 white cells were examined in every smear.

Figure 1 and Figure 2 from cases I and II show lymphocytes with vacuoles and some

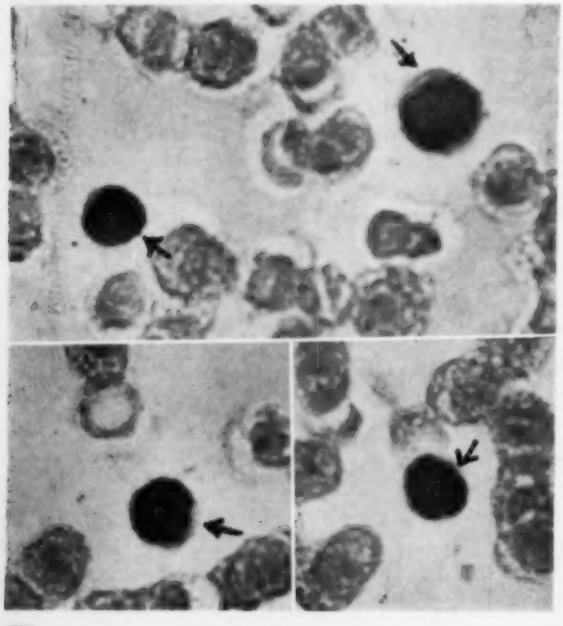

Fig. 1.—Blood smear from Case I. May-Greenwald Giemsa stain; magnification 1,385.

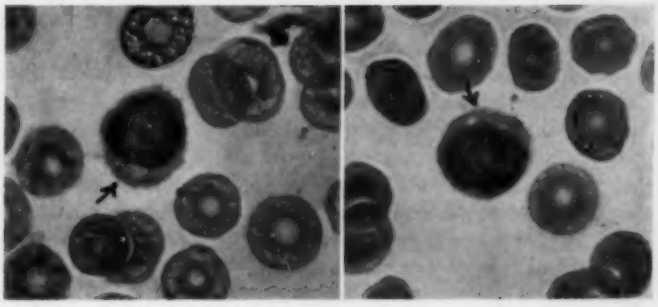

Fig. 2.—Blood smear from Case II.

light-reflecting inclusions in the protoplasm. Initially, the number of vacuolized cells was less than 5% of the total white cells. The average number of the vacuoles per cell was less than in the cases of Spielmeyer-Vogt's disease, contributing to the difficulty of finding v. l. in Tay-Sachs' disease. Four to 8 months later, the number of v. l. in Tay-Sachs' disease had increased to 13% and 8% respectively. In case IV, in an earlier phase of the disease, there were only 4% of v. l. Both parents in case I and the father in case II showed v. l. in the peripheral blood.

The finding of v. l. in Tay-Sachs' disease does seem appropriate, since this disease shares a disturbance of the lipoid metabolism in common with the juvenile form. It does appear, moreover, that the factor of the duration of the disease plays a role. Bagh and Hortling have shown a strict parallelism between the duration of the disease and the percentage of v. l. in their cases belonging to the juvenile form. In the Tay-Sachs form of the disease Aronson and his group have pointed out that the medical care now available to these patients has prolonged their life-span so that pathological changes can be observed which formerly escaped detection.[1] This prolonged duration of the disease may be one of the factors explaining the current observation of vacuolized lymphocytes in the peripheral blood of patients with Tay-Sachs' disease.

The parallelism between duration of the disease and number of the v. l. was not observed in Niemann-Pick's disease (Crocker and Farber).[5a] This could be explained not only by the physiological decrease of the lymphocytes in older children (Blackfan, Diamond and Leister)[5] but also by the primary pathological involvement of the lymphoid tissue characteristic of Niemann-Pick's disease.

Finally case numbers IV and V of our Tay-Sachs' disease group represent 2 couples each of whom lost a child as the result of Tay-Sachs' disease, 8 years and 3 years ago, respectively. This group is of special interest, since v. l. have been described only in the peripheral blood of the parents and siblings of the juvenile form.[20] In peripheral blood obtained from the parents of

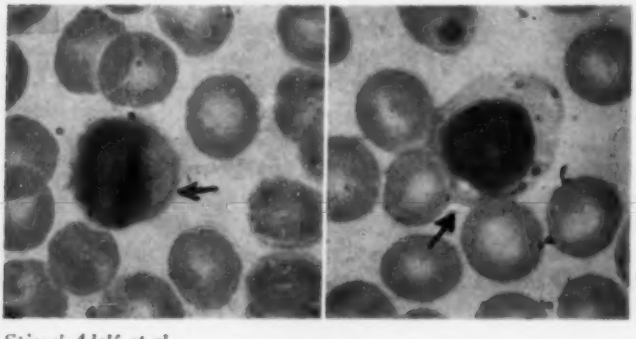

Fig. 3.—Blood smear from Case IV.

patient No. IV, up to 50% v. l. were found in the mother, and up to 30% in the father. Although we have no proof that these changes existed in the blood of the parents before the birth of the child, the number of the pathological cells when compared with the data of Bagh and Hortling[2] suggests that the phenomenon may be of 5-10 years duration. The lower values observed in case V correspond to the shorter duration. If these observations should be verified in more cases, the findings of v. l. in prospective parents could perhaps serve as a recommendation to prevent the conception of children doomed to Tay-Sachs' disease; (it is evident that other conditions capable of resulting in v. l. should be first excluded).

The next group comprises 4 children in the age group of 9-16 years affected with juvenile amaurotic idiocy (Table 5). The patients in this group were of non-Jewish origin. Blood smears of both the parents, and in 2 cases, of healthy siblings were available.

TABLE 5.—*Spielmeyer-Vogt's Disease*

Case No.	Sex	Age	% Vacuol. Lymphocytes
VI	M	10	Patient, 4%; Father, 20%; Mother, 15%
VII	F	9	Patient, 8%; Father, 2%; Mother, 12%
VIII	F	10	Patient, 15%; Father, 2%; Mother, 15%; Siblings, 12, 8%.
IX	M	16	Patient, 5%; Father, 5-8%; Mother, 1-3%; Siblings, 22, 2-3, 20-25%.
X	M	41	Patient, 50%; Mother, 12%; Sibling, >50%; Niece, daughter of healthy sibling, 12%.

The first patient was a white male, born September 25, 1948, who since May, 1957 had had minor motor seizures and since November, 1957 had exhibited degenerative changes in the macula. He died in December, 1957. Inclusion bodies were observed in the brain, similar to those observed by Poser and Radermecker.[19] Inclusion bodies in late infantile amaurotic idiocy have been described first by Bielschowsky[4]; in Tay-Sachs' disease by Marburg,[15] and by Marchand et al.[16] This patient had about 4% v. l. in the peripheral blood. Both of the parents had v. l. in the peripheral blood; the mother approximately 15%, and the father 20%.

The second child of this group, a white female 9 years old, showed macular degeneration, mental retardation and convulsive seizures. The EEG showed marked, diffuse cerebral dysrhythmia. The lack of concentration in the audiometric test corresponded to an age group below 4-5 years. The peripheral blood contained about 6% v. l. Both the parents had v. l. in the blood but in different quantities; while the father had about 2%, the mother showed at least 12% of v. l.

Of the third child, a white female, 10 years old, we know only that the diagnosis of juvenile amaurotic idiocy had been established by a leading University hospital. The patient showed about 15% v. l. in the peripheral blood. While the father had about 2%, the mother had 15% v. l.; the two apparently healthy siblings, of 1 and 4 years of age, 12% and 8% respectively.

Of interest is family IX. It consists of a father, mother and a boy of 16 who, according to an independent diagnosis at several institutions suffers from the Spielmeyer-Vogt form of the disease. One brother had died 1 year ago with symptoms of the same disease complicated by diabetes mellitus. Two sisters and a younger brother are apparently healthy. The younger girl shows some emotional disturbance. In the family of neither parent was any similar condition apparent. Direct inquiry revealed an English-Scotch ancestry on one side and on the other, six generations of New England and English background. A careful examination of the blood smears gave positive findings as to v. l. in both the parents, 5-8% and 1-3% respectively; in the sick boy 5%, and in the siblings, 22%, 2-3% and 20-25% respectively. Thus, contrary to the general concept, there exists a non-Scandinavian strain of Spielmeyer-Vogt's disease in this country which does show v. l.

The last case to be reported refers to a patient who showed a combination of different disease. He is one of six siblings. While the surviving mother and 3 children

apparently are in good health, 3 of the remaining siblings including our patient died after having shown cerebral degenerative changes for many years. The autopsy in one of the siblings revealed a cerebral lipoidosis with ballooned ganglion cells and pigmentation between the cells. The diagnosis was a combination of Hallervorden-Spatz' disease with amaurotic family idiocy similar to the case reported by Jervis.[11] The first symptoms in our patient started at the age of 15, and he died at 41 years of age, while our studies were in progress. According to the age limit stated by Diezel,[6] this case would be more of the Kufs-Hallervorden type, but the optic atrophy and blindness better fit into the pattern of the juvenile form. The vacuoles were larger and greater in number per cell than in the other cases (Fig. 4). The preliminary autopsy report

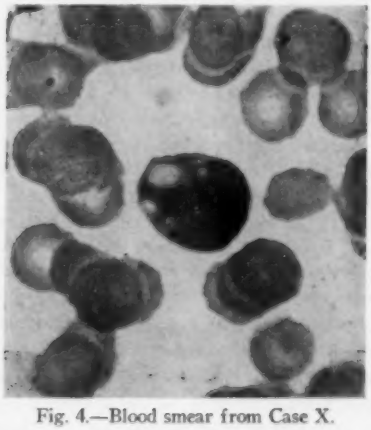

Fig. 4.—Blood smear from Case X.

by Drs. H. Riggs and A. Silverstein described a widespread lipoidosis of the ganglion and glial cells of the brain. Both the mother and the patient had a very large number of v. l. (12% and more than 50% respectively).

For an understanding of the results presented here it might be interesting to consider the role of the circulating lymphocytes. In his critical presentation, Naegeli [17] points out that the lymphocytic forms with large protoplasm occurring in children, and under pathologic conditions, represent older cells with phagocytic capacity. According to Bergell,[3] they are capable of secreting lipases. Such lipases acting upon intracellular fat globules could produce the vacuoles and explain the findings of vacuolized lymphocytes in the lipoidoses. Blackfan, Diamond and Leister [5] on the other hand, assume that the vacuoles observed in monocytes and lymphocytes in Niemann-Pick's disease reflect the findings in the tissues throughout the body since with appropriate stains they were found to be fat-laden. We have discussed disease conditions under which v. l. are observed at the beginning of this paper. V. l. alone, without determinations of their original content are, therefore, not specific for any form of lipoidosis. If the presence of other diseases causing v. l. can be excluded however, vacuolized lymphocytes can probably be of diagnostic value in amaurotic family idiocy. It is of interest that the findings of v. l. in children affected by Tay-Sachs' disease and demonstrable in their parents, removes one of the fundamental differences between the infantile and the juvenile form, although the scarcity of Tay-Sachs' disease data prohibits genetic evaluation at present.

In one respect, our findings are in agreement with the results of other investigators confirming genetic differences between Tay-Sachs' and Spielmeyer-Vogt's diseases.[9a] In our cases of Tay-Sachs' disease the male patients had mothers who showed more vacuolized lymphocytes than did the fathers, while in the group with Spielmeyer-Vogt's disease the male diseased off-spring had fathers with a greater per cent of vacuolized lymphocytes, and the female patients were associated with a higher maternal v. l. incidence.

According to Sjögren,[21a] the heredity of amaurotic family idiocy is not sex-linked. If the validity of our observations should be confirmed on a larger scale, sex-linked factors, especially governing the extent of formation of vacuolized lymphocytes, require re-evaluation.

Summary

1. The cerebrospinal fluid lipase content in Tay-Sachs' disease is practically the same as in a number of other neurological diseases examined.

2. Paper electrophoresis reveals statistically significant differences between the albumin-globulin ratio of children affected with Tay-Sachs' disease and normal controls. No such difference could be detected in the lipid pherogram which showed groupings similar to that of normal serum lipids.

3. Vacuolized lymphocytes (v. l.) could be detected in the peripheral blood of 3 children afflicted with Tay-Sachs' disease, in the parents of one of these patients, in the father of another and in a sibling of a child who had previously died of Tay-Sachs' disease. V. l. were also found in the blood of two pairs of parents of deceased children who had had Tay-Sachs' disease.

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Serial Enzyme Studies of Serum and Cerebrospinal Fluid in Amaurotic Family Idiocy

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The diagnostic utility of enzyme level analysis in biologic fluids has only recently been explored on a broad basis.[1,2,3] Particular emphasis has been placed upon the circulating glycolytic and transaminating enzymes. Based upon the supposition that for each determinable enzyme, there exists a specified pattern of concentration within individual tissues, it has been suggested and experimentally demonstrated that a rise in the serum content of any particular enzyme is a reflection of necrobiosis in such organs characterized by high concentration of that enzyme. It is further presumed that the enzyme elevation in serum represents a liberation from affected tissues rather than an elaboration. This is strengthened by the observation that the rise in circulating enzyme parallels the period of active destruction of the specified tissue and that such enzyme tests are of no avail as a measure of previous or quiescent degeneration. This concept, exemplified by the term "chemical biopsy" has been applied to acute necrotizing or degenerative lesions of myocardium, liver, brain and skeletal muscle.[1,2,3,4,5,6] No organ specificity is claimed, but through the judicious interpretation of associated clinical information, a reasonable localization can be adduced.

Twenty-nine patients with infantile amaurotic family idiocy (AFI) and two patients with Niemann-Pick's disease (NPD) were studied by the procedures to be presently outlined. Generally, the tests were performed at monthly intervals for the entire span of hospitalization. The matrix of AFI classification, outlined elsewhere,[7] has been used as the background against which the resultant biochemical changes were projected, and, as in the case of clinical and radiographic studies of this disease, the phases (initially based upon pathologic criteria) were shown to be of considerable pertinence when applied to the enzymatic data. Phase I was designated as that period of AFI from the commencement of symptoms (generally 4-7 months of age) to the fourteenth month of illness; Phase II covered that span from the fifteenth month until the twenty-fourth month of the disorder; Phase III was assigned to that period beyond two years of recognized and determined disease.

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From the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn.

Methods

Aldolase activity in serum and cerebrospinal fluid (CSF) was determined by the colorimetric procedure of Sibley and Lehninger.[8] Glutamic oxalacetic transaminase activity in serum and CSF was obtained spectrophotometrically with the method of Karmen, Wróblewski and La Due.[3] Lactic dehydrogenase of serum and CSF was determined with a Beckman D. U. spectrophotometer at 340 mμ by techniques described by Wróblewski and La Due.[3] The values of serum and CSF phosphohexose isomerase were estimated by the method of Bodansky.[9] A modification of the King-Armstrong procedure was used for serum acid phosphatase. Techniques for enzyme levels in central nervous system (CNS) tissues were essentially the same as those used for the biologic fluids except for the steps required to preserve enzymatic activity.

Serum Enzyme Studies

The first of the enzymes analyzed was aldolase, a glycolytic enzyme involved in intermediate carbohydrate metabolism. There is a particularly high concentration of aldol-

ase in myocardium, liver and skeletal muscle.[10] In contrast, the CNS concentration is relatively low. Aldolase is normally found in human serum, in an approximate concentration of 5-10 units per cc. (average, 7.14 units per ml.). A prompt and significant elevation of this ferment has been noted in cases of primary myopathy, myocardial infarction and obstructive or parenchymatous hepatic disease.

In studying the effects of varying forms of muscle atrophy upon the level of this enzyme in dogs, it was noted that skeletal muscle wasting, secondary to disuse or upper motor neuron paralysis, had no determinable effect upon the serum level of the enzyme.[11] In contrast, a delayed but distinct hyperaldolasemia was produced following experimental disturbance or severance of the lower motor neuron or its axonal projection. A comparable hyperaldolasemia was also seen in patients in whom the lower motor neurons were in some fashion affected. A group of diseases, collectively designated as the progressive nuclear myelopathies and including amyotonia congenita, arthrogryposis multiplex congenita and progressive spinal muscular atrophy were shown to be associated with inconstant rise in serum aldolase. AFI was included in this group only in the context of its progressive anterior horn cell implication. A composite analysis of serially determined serum aldolase in this disease has shown normal levels during the initial months of the disorder. After approximately 8-12 months of determined disease, the serum aldolase was elevated, generally to levels of about 15 units per ml. This elevation persisted for periods of 3 to 12 months and then reverted to the normal range. The hyperaldolasemia coincided with the clinical emergence and development of skeletal muscle atrophy. An analysis of the clinical course and pathologic evolution of AFI fails to disclose any other occurrence which may reasonably account for the hyperaldolasemia.

A more pronounced hyperaldolasemia was evident in the cases of NPD studied, and probably related to the additive effects of skeletal muscle atrophy and hepatic involvement. Application of this test to patients with other extensive and progressive neural disturbances but characteristically lacking in involvement of the anterior horn cells (viz., diffuse sclerosis), showed no abnormalities in aldolase levels despite clinically obvious and pathologically corroborated muscle atrophy.

Glutamic oxalacetic transaminase (GOT), an enzyme related to the intracellular synthesis of glutamic and oxalacetic acid, is diffusely distributed in the tissues of the body with greater concentration in the liver and myocardial musculature. The relative concentration in CNS tissues, in contrast to aldolase, is high. An increase in the serum levels of this enzyme has been equated with myocardial or hepatic disease in the past. Recently some observers have noted a transient rise of serum and CSF GOT in clinically observed, as well as experimentally induced, instances of cerebral vascular disease.[12]

In a series of dogs, each weighing about 13 kg., injection of 1 gm. of fresh homologous cerebral homogenate resulted in a non-sustained hypertransaminasemia.[13] When a similar series of control animals was inoculated with a heat-inactivated homogenate, no elevation was manifest. From such results, as well as serial observations in cases of myocardial infarction in the human, it becomes apparent that the continuing elevation of this enzyme is dependent upon the duration of active necrosis of the appropriate tissues. Thus, this procedure is of no value in determining the existence of an old, cardiac infarction. In 29 cases of AFI, serial monthly serum GOT determinations were performed revealing an enduring and uniformly elevated level. In some of the children so tested, the hypertransaminasemia has been recorded for periods exceeding 30 months. This may be interpreted, therefore, as indicating necrosis of neural tissues for the major span of the disorder. The increase in serum GOT is much more marked in the initial months of the disease. Although wide monthly fluctua-

tions are evident, the hypertransaminasemia gradually declined in the ensuing years. In one protracted case, alive after 43 months of illness, the serum GOT has finally reverted to the upper limits of normal. If an attempt is made to extrapolate these findings in the reverse direction, the elevation of serum GOT seemingly predates the clinical recognition of the disorder. In one substantiated case, an ostensibly normal 4-month-old male infant (a sibling of a previous patient with AFI) was bronght to the clinic for routine examination. No neurologic abnormalities were determined and there was no evidence of macular degeneration. Serum GOT was unequivocally elevated (160 units per ml.) and 4 months later at the age of 8 months the child finally developed the characteristic symptoms of AFI. This case lends further credence to the contention that the morphologic changes of AFI prevail at birth, but do not penetrate clinically until the accumulating neurocellular storage process is sufficiently critical to depress or alter the functions of the neurons.

Similar elevations of serum GOT were noted in the cases of NPD studied. A representative group of infantile neurological illnesses, including diffuse sclerosis, cerebral palsy, convalescent poliomyelitis, hydrocephalus and congenital anomalies of the nervous system, were also surveyed. The serum level of GOT remained essentially normal in all instances.

Serum lactic dehydrogenase (LD) has been shown to be elevated in some neoplastic states, particularly leukemia, by Wróblewski and co-workers.[14] A significant rise in the serum values of this enzyme has been noted in AFI and (in approximate parallelism to the serum GOT response described above) is more severely elevated in the first phase of the disease. No other primary neurologic disorder so tested, *including* NPD, has shown any abnormalities in serum LD.

Serum acid phosphatase, reported by some observers as elevated in NPD,[15] and serum phosphohexose isomerase were determined in a limited number of children with AFI. No abnormal results were obtained.

Cerebrospinal Fluid Enzyme Studies

Comparable procedures were applied to CSF. In many instances, the CSF and serum samples were withdrawn simultaneously (Fig. 1). CSF aldolase was significantly elevated in the 14 cases of AFI studied. In contrast to serum aldolase where the elevation was restricted approximately to the second year of disease (coincident with the establishment of skeletal muscle atrophy), the spinal fluid rise was more prominent at the onset of the disease

TABLE 1.—*Serum and Cerebrospinal Fluid Levels in Central Nervous System Lipidoses*

	Inf. Amaurotic Fam. Idiocy			Neimann-Pick's Disease			
	No. Pts.	No. Determ.	Level *	No. Pts.	No. Determ.	Level *	Normal Level
Serum			†				
Aldolase	29	186	10.7 (3.7–29.2)	2	15	10.2 (5.5–19.1)	5–10
G. O. transaminase	29	171	99 (41–300)	2	17	183 (90–396)	22–40
Lactic dehydrogenase	16	39	1051 (480–1960)	2	5	430 (260–658)	200–680
Cerebrospinal Fluid							
Aldolase	14	44	1.7 (0.1–5.7)	2	5	3.9 (1.4–7.4)	0–1
G. O. transaminase	14	44	42 (22–94)	2	5	31. (25–41)	4–14
Lactic dehydrogenase	14	26	113 (30–212)	2	2	60 (45–75)	5–40

* Arithmetic mean, and, in parentheses, range of values.
† Units/ml.

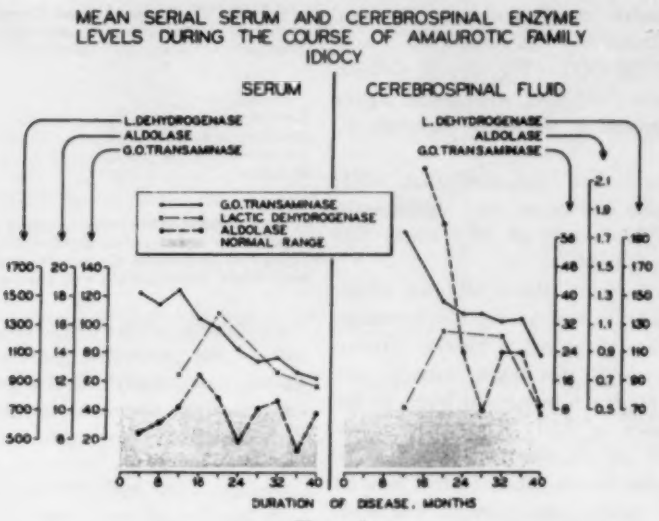

Figure 1

and tended to regress as the course of the disorder progressed.

In the initial months of the disease, values averaging 3.6 units per ml. of aldolase were recorded (normal range, 0-1 units per ml.). The apparent absence of any parallelism between serum and CSF aldolase suggested the possibility that the changes in each of the two biologic fluids were in response to different pathophysiologic components of AFI.

CSF GOT was elevated throughout the course of AFI but higher in the initial months. A distinct concordance between CSF aldolase and GOT was therefore evi-

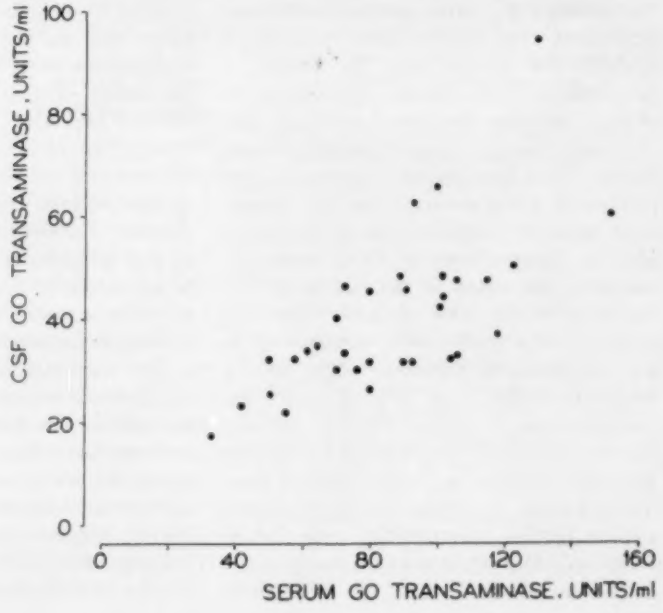

Fig. 2.—On 33 separate occasions, in 14 patients with amaurotic family idiocy, simultaneous serum and cerebrospinal fluid specimens were obtained. G. O.-transaminase values in the paired specimens were plotted in the graph above. Note the approximate linear proportion of levels within the two biologic fluids.

dent. A further correspondence was seen in the simultaneously determined CSF and serum levels of GOT (Fig. 2). A distinct rise in spinal fluid LD was noted, again more pronounced in the early segments of the illness.

Preliminary data indicates that CSF phosphohexose isomerase was significantly elevated in the 6 cases of AFI where this procedure was used.

In general terms, the CSF and serum GOT and LD as well as the CSF aldolase and isomerase displayed a similar pattern marked by peak elevations initially and asymptotic approach to normal levels in the chronic phases of AFI. The serum aldolase pursued an independent response apparently more closely allied to the induced atrophy of skeletal musculature. The CSF enzyme levels in NPD were qualitatively similar to those displayed in AFI with the notable exception that LD was invariably normal in the former disease.

Central Nervous System Tissue Enzyme Studies

Two children with AFI, in the series under discussion, succumbed shortly after the technique for tissue enzyme levels were established. The first of these cases was a 43-month-old female and the second, a 39-month-old male infant. Control levels of tissue enzymes were determined in a child of comparable age, dying of congenital heart disease. The quantitative procedures were performed simultaneously and all precautions taken to minimize loss of enzymatic activity. Tissue levels of GOT were significantly decreased in all levels of the neuraxis, in the cases of AFI. The loss, however, was much more pronounced in the supratentorial segments where activity tended to be less than 50% of anticipated concentration. A similar disparity between infratentorial and supratentorial LD activity was also observed in tissues derived from the AFI cases. A curious finding, which will require further substantiation, was the *increase* in LD levels within the medulla and cerebellum of the 2 cases of AFI. Since infratentorial atrophy is a conspicuous feature of the protracted phase of AFI [7] this finding may merely reflect a greater relative concentration incident to the involution rather than any true elevation (Table 2).

TABLE 2.—*Brain Tissue Enzyme Levels, Amaurotic Family Idiocy*

	GOT	LD
Cerebral white matter	25% *	39%
Cerebral gray matter	49%	97%
Cerebellum	79%	235%
Medulla	85%	134%

* Data, based upon two autopsied cases of AFI, are expressed as percentage deviation from normal. Normal values were obtained from brain tissues of a male infant dying of congenital heart disease. Determinations were performed simultaneously.

Comment

A number of consistent alterations have been recorded in serum and CSF enzyme levels during the evolution of amaurotic family idiocy (AFI). Such elevations represent some of the direct and indirect consequences of continuing, diffuse ganglionic storage characteristic of the disease. The impermanent serum aldolase rise reflects the attending neuromuscular atrophy. The hyperaldolasemia has been tentatively attributed to the secondary muscular atrophy rather than the more fundamental neuronal degeneration in view of normal serum aldolase values during the period of greatest cerebral breakdown (as determined by release of enzymes into the CSF), and the approximate co-existence of the subsequent serum aldolase rise with skeletal muscle wasting. Further, since the aldolase content of muscle is almost five-fold greater than in brain, breakdown of the former tissues would be a more influential factor in determining serum levels of this enzyme.

The sustained serum GOT elevation in AFI and Niemann-Pick's disease (NPD) is probably the direct consequence of cerebral necrosis. The cerebral lipidoses of infancy, therefore, constitute the only primary intracranial disease associated with prolonged hypertransaminasemia. Studies involving serum LD show results essentially similar to those noted with GOT.

TABLE 3.—*Serum and Cerebrospinal Fluid Enzyme Levels in Various Neurological Diseases of Childhood*

	Serum			Cerebrospinal Fluid		
	Aldolase	GOT	LD	Aldolase	GOT	LD
C. N. S. anomalies	–	–	–	–	–	–
Cerebral palsy	–	–	–	–	–	–
Diffuse sclerosis	–	–	–	–	–	–
Poliomyelitis, convalescent	–	–	–	–	–	–
Acute meningitis	+	–	–	+	+	–
Neimann-Pick's disease	++	++	–	++	++	–
Amaurotic family idiocy, infantile	++	++	++	++	++	++
Amaurotic family idiocy, juvenile	–	–	–	–	–	–

+ = Elevation noted occasionally during the course of the designated disease.
++ = Significant elevation consistently noted during the course of designated disease.

The alterations of enzyme activity in CSF appear to be more accurate determinants of the existence and stage of the disease, than the changes in serum. None of the enzyme changes, individually, can be construed as pathognomonic of AFI. The results, however, do suggest that nervous system breakdown incident to the lipidoses may be distinguished from other types of cerebral degeneration by the collective determination of multiple enzyme levels in the biological fluids (Table 3).

It is of interest to note that no serum or CSF enzymatic abnormalities have been noted in the generically allied juvenile amaurotic family idiocy. Within the family of *infantile* CNS lipidoses, a point of possible diagnostic distinction appears to arise between AFI and NPD. While the aldolase and GOT changes within the serum and CSF appear to be indistinguishable in these two illnesses, a distinct rise in serum and CSF LD is found only in the cases of AFI.

The CNS *tissue* enzyme levels are significantly depressed in AFI suggesting again that the biologic fluid enzyme elevations represent a nonspecific liberation from affected tissues. The topographic extent of enzyme loss in intracranial tissues of cases of AFI, however, is not uniform. The sites of more recent developmental appearance (viz., neopallium) show a far greater depression of enzyme activity than the more archaic structures (viz., cerebellum, medulla). Such findings offer further verification of morphologic data indicating that a distinct hierarchy of involvement exists in AFI based upon a scale of phylogeny.[7]

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The Partition of Tissue Phospholipides by Phosphorus Analysis

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The known tissue phospholipides may be divided into four different groups on the basis of the nature of certain bonds between the lipide groups and between the phosphorus-containing groups of their molecules: 1. *Ester phospholipides* such as lecithin (phosphatidyl choline), certain cephalins such as inositol cephalin, phosphatidyl ethanolamine, and phosphatidyl serin. Their lipide groups are exclusively fatty acids which are linked to the phosphorus-containing moiety of each molecule by alkali-labile ester bonds. 2. *Plasmalogens.* Each molecule of this group contains as lipide groups an aldehydogenic group and a fatty acid group. The former appears to be attached to the phosphorus-containing moiety in the form of a vinyl ether (dehydrated hemi-acetal)* by a linkage which is very labile towards acids and mercury[2] ions, the latter by an alkali-labile ester linkage.[1,13,14] 3. *Ether phospholipides*[3] which contain as lipide groups long-chain alcohol groups which are attached to the phosphorus-containing part of their molecule by alkali- and acid-resistant ether linkages. 4. *Sphingophospholipides* such as sphingomyelin which may be defined as esters between phosphoryl choline and a ceramide group. Both the phosphoryl ester bond between these groups as well as the amide bond between the fatty acid and the sphingosine moiety of the ceramide group are resistant toward alkali.

The different conditions required for the cleavage of the bonds between the lipide groups and the phosphorus-containing constituents of the various types of the phospholipides suggest the possibility of developing hydrolysis procedures for the quantitative differentiation between the phospholipide components present in lipide extracts of tissues. Such a method had been described several years ago by Schmidt et al.[4] for the quantitative partition of phospholipides into the sum of ester phospholipides and plasmalogens on the one hand, and into that of

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* The nomenclature used in this paper for the individual lipide fractions follows the suggestions of Rapport et al.[1] The term "aldehydogenic group" defines the group responsible for the positive Schiff reaction of certain lipide fractions. The term "plasmalogen" which had been introduced by Feulgen and Voit,[2] designates the genuine, non-saponified, aldehydogenic phospholipides, while the terms "acetal phospholipides" or "saponified plasmalogens" or "lysophosphatidals" are applied synonymously to their derivatives obtained by removal of the fatty acid groups by saponification. The term "mercury-labile lipide phosphorus" is used for the difference between the amounts of water-soluble phosphorus obtained after treatment of lipide emulsions (*a*) with alkali, and (*b*) with mercury salts and subsequently with alkali.

The term "sphingophospholipide fraction" is occasionally used for the sum of sphingomyelins and ether phospholipides.

the ether- and sphingophospholipides on the other hand. For this separation, aqueous emulsions of the extracted lipides were first treated with N sodium hydroxide at 37° in order to transform all phosphoryl groups of the ester phospholipides into water-soluble phosphorus compounds, and subsequently with an excess of trichloroacetic acid in order to cleave the aldehydogenic linkages of the plasmalogens. The amounts of total phosphorus in the water-clear filtrate obtained from the samples after the treatment with trichloroacetic acid was interpreted as the phosphorus of the sum of ester-phospholipides and plasmalogen, those remaining in the lipide precipitates as the phosphorus of the sum of the ether- and the sphingophospholipides.

A serious disadvantage of this procedure —and of all analytical methods involving plasmalogens or acetal phospholipides—is the fact that all information concerning their behavior toward hydrolyzing agents is based on aldehyde determinations. Since all tests developed for the quantitative assay of lipide aldehydes require acidity conditions at which extensive liberation of the aldehydogenic groups of plasmalogens occurs, such tests furnish only indirect information regarding the conditions required for complete cleavage of bound lipide aldehyde groups by a given hydrolyzing agent. In the procedure described above, incubation of the saponified lipide emulsion with trichloroacetic acid was assumed to produce quantitative liberation of the bound aldehyde groups because these conditions corresponded in regard to acidity, incubation time, and temperature to the conditions required for maximal color development in the Schiff test.

A reexamination of the validity of this assumption was all the more desirable as Brante [5] as well as Edgar [6,7,8] reported that cephalin fractions obtained from brain and spinal cord contained appreciable amounts of an alkali-resistent phospholipide which was different from sphingomyelin, and which was tentatively designated as "cephalin B." Since all cephalin samples which have been isolated from plasmalogen-containing lipide mixtures are contaminated with appreciable amounts of plasmalogens, and since cephalins and plasmalogens cannot be separated at present, it seemed possible that the conversion of the plasmalogen phosphorus to water-soluble phosphorus compounds was incomplete under the conditions used in the procedure of Schmidt et al.[4]

The most important reason for the reinvestigation of the hydrolysis of plasmalogen, however, was the observation by Schmidt et al. who reported in 1951 [9,10] that the crystallized acetal phospholipides isolated from muscle by Feulgen and Bersin,[11] and from brain by Thannhauser, Schmidt, and Boncoddo [12] yielded water-soluble phosphorus when incubated at an acidity similar to that of the Schiff test, whereas the original lipide mixtures used as starting materials were not converted to water-soluble phosphorus compounds under comparable conditions. In contrast to the striking difference in the behavior of the phosphoryl groups, both genuine and purified samples showed closely similar extinctions in the Schiff tests. Schmidt postulated, therefore, that genuine acetal phospholipides (plasmalogens) differed by the presence of a second lipide group in their molecule from the crystalline acetal phospholipides. This conclusion was borne out by subsequent independent investigations of Klenk et al.,[13] Debuch,[14] and Rapport et al.[1] The use of phosphorus determinations for the study of the hydrolysis of plasmalogens was only possible if the application of trichloroacetic acid for the separation of lipides and water-soluble compounds could be replaced by a procedure which affected this separation within the stability range of the acid-labile plasmalogens. It was found that half-saturation of aqueous lipide emulsions with ammonium sulfate at pH 5.5, in analogy to Lowry's [15] use of this reagent for the deproteinization of solutions containing acid-labile phosphoric acid compounds, was suitable for this purpose.

The use of this reagent made it also possible to obtain separately the values of the ester phospholipides and of the plasmalogens

by phosphorus determination, and to determine in one gram of tissue the amounts of ester phospholipides, of plasmalogens, and of the sum of the ether and sphingophospholipides. The description of this procedure and of the evidence supporting the interpretation of the analytical data will be given in this paper. Some representative values obtained by the application of the method to tissue analysis will also be reported.

Materials and Methods

Cephalin was prepared from beef brain according to Folch-Pi [16] and stored at −16°. The total fraction as well as the phosphatidyl ethanolamine fraction (fraction 5) was used. *Lecithin* was prepared from egg yolk according to Hanahan and Jayko,[17] *sphingomyelin* from lung according to Thannhauser et al.[18]

Feulgen Reaction (Modified procedure of Feulgen and Gruenberg [19]). A measured portion of an aqueous emulsion of tissue phospholipides (see experimental section) was brought to a volume of 7 ml. in a test tube (8×1) and acidified by the addition of 1 ml. of glacial acetic acid. Acidification prior to the addition of Schiff reagent was found essential since the latter decomposed spontaneously under formation of a red color at neutral or alkaline reaction. 0.5 ml. of a 5 per cent aqueous solution of mercuric chloride, and 10 ml. of the reagent described by Feulgen and Gruenberg were then added to the sample.[17] The test tube was tightly stoppered with a rubber stopper, and the reaction mixture was vigorously shaken for 30 seconds. The formation of the insoluble colored reaction product was practically completed during 2.5 hours at room temperature. It was extracted with 20 ml. of *n*-amyl alcohol (Baker's analytical grade). After separation of the two layers, a portion of the amyl alcohol layer was clarified in a small angle centrifuge. The extinction was determined at 555 mμ in a Beckman Model B spectrophotometer. This procedure will be designated as Feulgen-technique No. 1 in the following sections of this paper. With a given lipide emulsion the extinctions were strictly proportional to the amounts of lipide emulsion at least up to amounts of 1μ mole or plasmalogen. We are deeply grateful to Dr. Maurice M. Rapport, Department of Biochemistry, Yeshiva University School of Medicine, for determination of the molar extinction of aldehydogenic lipides on the basis of measurements carried out on a sample of pure, crystallyzed lysophosphatidal ethanolamine from beef muscle.[1] The molar (based on phosphorus analysis) extinction of this standard compound was found to be 23,000.

Remarks Concerning the Technique of the Feulgen Reaction on Lipides

1. *Filtration of colored reaction product prior to colorimetry.*—As pointed out by Feulgen et al.[17] the colored condensation product of fuchsin-sulfurous acid and of long-chain lipide aldehydes retains the solubility properties of lipides. Filtration of the reaction mixture over a thin layer of infusorial earth (e. g. Hyflo Filter Aid, John Manville Company Inc., Boston, Massachusetts) on a small Buchner funnel yields a colorless filtrate whereas most (but not all) of the pigment can be extracted immediately from the filter cake by suspending it in amyl alcohol. Furthermore, when the bulk of inert lipides of the analyzed sample is increased by adding 10 mg. of sodium stearate in aqueous solution prior to the addition of the other reagents required in the Feulgen reaction, the pigment is present exclusively in the lipide precipitate that forms immediately on addition of the acetic acid and floats on top of the reaction mixture leaving a colorless bottom layer. It can be easily filtered off by gravity on a Whatman No. 1 filter. After washing the precipitate with a solution of sulfurous acid which was prepared according to the directions given for the preparation of Feulgen reagent with omission of the fuchsin, the precipitate can be extracted with 20 ml. of amyl alcohol. The extinction of the clear extract can be used for the quantitative evaluation of the Feulgen reaction (Feulgen

technique No. 2). Under comparable conditions, the values obtained with this procedure are 88% of those obtained with the standard method (Feulgen technique No. 1) described above. Filtration of the product of the Feulgen reaction prior to its extraction with amyl alcohol was found to be a useful modification of the standard procedure when the available samples of amyl alcohol gave blank extinctions with Feulgen reagent since, usually, the contaminations responsible for this behavior could not be eliminated by simple purification procedures such as distillation of the amyl alcohol after treatment with sodium bisulfite.

Presence of more than trace amounts of proteins in the lipide emulsion prevents the extraction of the reaction product of the Feulgen test by means of amyl alcohol.

2. *Direct colorimetry of aqueous reaction mixture.*—The concentrations of total lipides required for the Feulgen reaction in emulsions containing aldehydogenic lipide groups in abundance (such as brain, spinal cord or heart) are so small that the dispersion of the pigment remains for many hours fine enough to permit direct colorimetry of the aqueous suspension. The maximum of the extinction is at 580 mμ under these conditions, and the molar extinction at this wave length is the same as that of the corresponding amyl alcohol extract at 555 mμ. The extinctions were corrected by subtracting those of control tests with sulfurous acid in absence of fuchsin (Feulgen technique No. 3).

Attempts to use an anionic detergent (lauryl sulfate) as solubilizer of the product of the Feulgen reaction had to be abandoned because lauryl sulfate itself slowly gave a positive Schiff test of considerable intensity even after five recrystallizations.

Experimental

Procedure of Phospholipide Partition.—Principle: An aqueous emulsion of the extracted tissue lipides is divided into three portions. Sample A serves for the determination of the total lipide phosphorus. In sample B, the phosphoryl groups of the ester phospholipides are quantitatively converted to water-soluble phosphorus compounds by shaking the emulsion in N sodium hydroxide for 18 to 20 hours at 37°. In sample C, the aldehydogenic groups of the plasmalogens are quantitatively hydrolyzed by shaking the emulsion in presence of 1 per cent mercury (2) acetate at pH 5.5 for one hour at room temperature. Subsequently, the fatty acid groups of the plasmalogens as well as those of the ester phospholipides are quantitatively liberated by shaking the emulsion in N sodium hydroxide for 18 to 20 hours at 37°.

The water-soluble degradation products of the saponified samples B and C are separated from the lipides by approximately 0.5 saturation with ammonium sulfate at pH 5.5. (At this acidity, the aldehydogenic bonds of the saponified plasmalogens of sample B are not hydrolyzed to a measurable degree.) The coagulated suspensions B and C yield water-clear filtrates in which the amounts of total phosphorus are determined. The phosphorus value of sample B represents the amount of the ester phospholipide phosphorus, the difference between the phosphorus values of sample C and B the amount of the plasmalogen phosphorus, that between the phosphorus values of samples A and C the sum of the phosphorus values of the ether and of the sphingophospholipides.

Reagents: Ethanol (95%)—ethyl ether mixture (75/25 v/v)
Ether petroleum (B. P. 30° to 60°)
8 N aqueous sodium hydroxide solution
A 12 per cent solution of mercury (2) acetate to which 12 gm. sodium (crystalline) is added.
The solution is prepared freshly before it is used.
Ammonium sulfate CP-grade
5 N sulfuric acid
2.5 per cent solution of ammonium molybdate.
Solution of 1-amino-2-naphthol-4-sulfonic acid.
(Prepared according to Fiske and Subbarow.[20])

Procedure: Preparation of Aqueous Emulsions of Tissue Phospholipides.—The lipides are ex-

tracted by refluxing between 1 and 10 gms of moist tissue for 10 minutes with 20 volumes of the alcohol-ether mixture. The extract is brought to dryness under reduced pressure at 40° bath temperature, preferably in a flash evaporator, and the residue is reextracted with ether petroleum. The lipide solution is centrifuged in a stoppered tube at room temperature and concentrated to dryness under reduced pressure. The dry lipides are dissolved in a small amount of ether, and the solution is brought to dryness under reduced pressure. The extraction with ether and the removal of the solvent are repeated two times with the purpose of removing as completely as possible the small quantities of ether petroleum which always remain with the lipides after the first distillation. For the emulsification in water, the dry lipide residue is dissolved in the distilling vessel in minimal amounts of 95 per cent ethanol on a boiling water bath. (The amounts of alcohol depend on the volume of the subsequent aqueous emulsion and should not exceed 10 per cent of this volume.) When the lipides are completely dissolved, 3 to 4 ml. of water for each gram of extracted tissue are rapidly added under vigorous shaking. A very stable emulsion forms, particularly easily with lipides of the nervous system and of liver. The volume is adjusted to 10 ml for each gram of extracted tissue, and the emulsion is homogenized in a tephlon homogenizer. While the volume conditions suggested in this description might be altered according to the lipide concentration in the tissues it is advisable to keep the volumes of the lipide emulsions as small as feasible. Emulsions whose volumes are smaller than 10 ml. are prepared by performing the evaporation and emulsification of the lipides in distillation vessels of the shape of test tubes rather than in round-bottom flasks.

A. Determination of Total Lipide Phosphorus. A sample of the lipide emulsion is diluted five fold, and the total phosphorus is determined in a suitable portion of the dilute emulsion according to the method of Fiske and Subbarow.[20]

B. Saponification of the Ester Phospholipides. A suitable portion of the lipide emulsion (e. g., 4 ml. of an emulsion of the lipides of 1 gm. of spinal cord in 10 ml) is pipetted into a 10 ml volumetric flask, and mixed with 0.8 ml of water and 1.2 ml of 5N sodium hydroxide. The flask is tightly stoppered and shaken in a mechanical shaker for 20 hours at 37°. According to recovery experiments with added known amounts of egg yolk phospholipides, this temperature is necessary for the complete saponification of the ester phospholipides.

C. Hydrolysis and Saponification of Plasmalogens.—A third sample of the emulsion is prepared for the cleavage of the plasmalogens to water-soluble phosphorus compounds. Since the phosphorus of the plasmalogens is obtained by difference between the values of C and B, the cleavage of the plasmalogens is preferably carried out simultaneously with the saponification of sample B and with the same amount of lipide emulsion used for this saponification. A 4 ml portion of the lipide emulsion is mixed in a 10 ml volumetric flask with 0.8 ml of mercuric acetate solution. The mixture is mechanically shaken at room temperature for one hour. After addition of 1.2 ml of 5 N sodium hydroxide, the saponification is carried out as described for that of sample B.

Remark: Accurate measuring of samples A, B, and C is obviously of critical importance in view of the differential nature of the partition method.

Separation of Water-Soluble and Lipide Phosphorus Compounds in the Saponified Samples.—The separation of the water-soluble phosphorus compounds from the phospholipides is a critical step in the procedure. Since one of the purposes of the method is the separate determination of the ester phospholipides and of the plasmalogens this step has to be achieved at neutral or weakly acid pH owing to the unusual sensitivity of the aldehydogenic group towards acids. It was found that even transitory acidification during the neutralization of the saponified B-samples with mineral acids resulted in faulty elevation of the ester phospholipide values because of partial hydrolysis of the acetal phospholipides. The necessity to maintain weak acidity during the operation in presence of large concentrations of fatty acids is the reason why all attempts to achieve extraction of the saponified lipides with organic solvents were unsuccessful. The only procedure that resulted in the complete removal of the lipides from the aqueous phase without liberation of lipide aldehydes was half-saturation with ammonium sulfate at pH 5.5, in analogy to the application of ammonium sulfate for deproteinization at weakly acid reaction which had been introduced by Lowry et al.[18]

Of each of the saponified samples B and C, 5 ml.-portions are pipetted into 10 ml. volumetric flasks. After addition of 3 gm. of solid ammonium sulfate and of 0.5 ml. of glacial acetic acid to each of the respective samples, the mixtures are gently shaken until

the ammonium sulfate is almost completely dissolved, and adjusted to a volume of 10 ml. Formation of some foam is prevented by addition of a drop of octyl alcohol if necessary. The mixtures are filtered immediately by gravity through small Whatman No. 1 paper which yields water-clear filtrates almost invariably. In the very few cases of slightly turbid filtrates clarification was always achieved by repeating the filtration through Whatman No. 42 filters.

Ashing of the Lipide-Free Filtrates for the Determination of Total Phosphorus.—The conversion of the phosphoryl groups of the water-soluble degradation products of the phospholipides to orthophosphoric acid is carried out by ashing with sulfuric acid and hydrogen peroxide in the usual manner. Owing to the high concentration of ammonium sulfate in the filtrates, the wet combustion of a 2 ml. portion of each filtrate requires ashing with 2.5 ml. of 5 N sulfuric acid.

Removal of Mercury (2) Ions from the Ashed Liquids of the C-Samples.—While the presence of mercury (2) salts does not interfere with the colorimetric phosphorus determination when the extinctions of 625 mμ exceed the value of 0.3, it produces slight turbidities at lower concentrations of phosphoric acid. For the elimination of the mercury (2) salts, the ashed liquid is quantitatively transferred to a volumetric flask of 25 ml. capacity. Hydrogen sulfide is bubbled through the sample, and its excess is removed by aeration. After rinsing the gas-inlet capillary with water, the volume of the suspension of the mercury sulfide is adjusted to the mark of the volumetric flask, and the sulfide is removed by gravity filtration through a small dry filter. An aliquot of the filtrate is used for the determination of the total phosphorus after the necessary adjustment of the sulfuric acid concentration.

Determination of Phosphorus.—Direct application of the method of Fiske and Subbarow [20] to the ashed samples is feasible provided that the quantities of the analyzed phospholipide fractions are sufficient to yield reasonably high extinctions in the phosphorus determination in a volume of 25 mls. Under the volume conditions described in the preceding paragraphs, this requires phospholipide quantities occurring in 0.5 gms. of fresh brain or spinal cord. The main reason for the necessity of relatively large quantities of starting material for the analytical partition of the phospholipides is the large proportion between the amount of sulfuric acid and that of the phosphorus-containing sample of filtrate which is necessitated by the large concentration of ammonium sulfate, and which requires an unfavorably high dilution for the final colorimetry.

Observations Pertinent to the Validity of the Procedure

1. *Quantitative Cleavage of the Fatty Acid Ester Bonds by N Sodium Hydroxide at 37°.*—When emulsions of the total lipide fraction of brain or of spinal cord were saponified with N sodium hydroxide under the conditions described above, the hydroxamic acid test of Rapport and Alonzo [21] for esterified fatty acids became negative. (The fatty acid amide bond in sphingomyelin does not give a positive hydroxamic acid test according to experiments with pure sphingomyelin.)

Furthermore, saponification of the lipides of rat liver and of egg yolk yielded amounts of water-soluble phosphorus which ranged between 95 and 98 per cent of the total lipide phosphorus. Since only very small amounts of plasmalogens and of sphingophospholipides occur in these tissues according to methods based on aldehyde and choline determinations, this shows that all fatty acid ester bonds of the phospholipides were cleaved during the incubation with N sodium hydroxide. Recovery experiments in which the lipide emulsions prepared from mixed solutions of known amounts of the total lipides of brain and of egg yolk were saponified by N sodium hydroxide under the conditions described above yielded ester phospholipide values which agreed quantitatively with those calculated from the sum of

TABLE 1.—*Extent of Saponification of Ester Phospholipides at Various Relative Volumes of N Alkali Solution*

Ml of Lipide Emulsion *	Ml of Saponification Mixture †	Ester Phospholipides Mg P per 100 Gm of Moist Spinal Cord
10	35	142
10	50	137
10	100	144

* Containing the lipides of 2 gm of beef spinal cord in 10 ml.
† All samples adjusted to a final concentration of N sodium hydroxide.
Saponification time 18 hours at 37°.

the ester phospholipides determined separately in the component solutions of the mixture.

The amount of N sodium hydroxide used in the procedure for the complete saponification of the ester phospholipides was 5 ml. for the lipides of 1 gram of moist tissue. The results reported in Table 1 show that the values of the water-soluble phosphorus remain essentially unchanged by using larger relative volumes of alkali.

Time Course of Saponification of Ester Phospholipides by N Sodium Hydroxide at 37°: The time course of the conversion of the phosphoryl groups of ester phospholipides to water-soluble phosphorus compounds in N sodium hydroxide at 37° is shown in Figure 1. After a rapid increase of the amounts of water-soluble phosphorus during the first two hours, the hydrolysis proceeded at slower rates until the amounts of water-soluble phosphorus reached the maximal value after 18 hours. The rapid initial rates of saponification are in agreement with observations of Dawson [22] on the effect of methanolic potassium hydroxide on ester phospholipides. If the saponification was carried out in Pyrex flasks, no material yielding a blue color with the Fiske-Subbarow reagent was released from the glass during this period. Extension of the saponification beyond 20 hours, however, resulted in progressive liberation of small, but measurable quantities of such substances (presumably silicates) from the glass.

Yields of Water-Soluble Phosphorus at Various Temperatures of Saponification of Total Tissue Phospholipides: The effect of temperature on the yield of water-soluble phosphorus compounds formed during the saponification of the total phospholipides of spinal cord is shown in Table 2. Whereas saponification at 37° increased this yield by 17 per cent over the amount of water-soluble phosphorus released at room temperature, a further increase of the temperature to 50° resulted in an additional liberation of water-soluble phosphorus of only 2 per cent.

Since the phosphorus of lipide mixtures whose phosphatides consist almost exclusively of ester phospholipides (lipides of liver and egg yolk) was practically completely converted to water-soluble phosphorus by a 20-hour saponification at 37°, it is likely that the small increase of water-

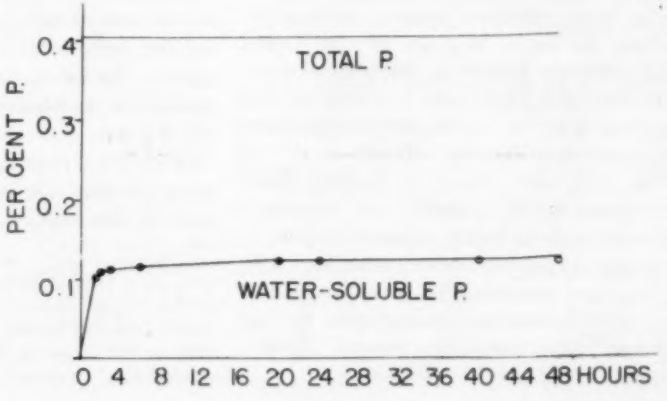

Fig. 1.—Formation of water-soluble phosphorus compounds from phospholipids during incubation in aqueous N sodium hydroxide at 37°. The P-values are expressed as per cent P of fresh tissue. The lipide emulsion contained the total lipides of 4 gms of beef spinal cord in 50 mls of N sodium hydroxide.

TABLE 2.—*Yields of Water-Soluble Phosphorus Compounds During Saponification of Lipides of Spinal Cord at Various Temperatures*

Temperature of Saponification (N Sodium Hydroxide, 20 Hours)	Water-Soluble Phosphorus (Mg per 100 Gm of Fresh Tissue)
25°	125
37°	146
50°	140

soluble phosphorus which was observed when the saponification temperature of lipide emulsions of spinal cord was raised from 37° to 50° was caused by a small degree of degradation of sphingomyelin.

II. *Stability of Aldehydogenic Groups of Plasmalogens During Preparation of Lipide-Free Filtrates.*—The application of specific hydrolysis techniques for the separate determination of ester phospholipides and plasmalogens required a criterion for the stability of the latter under the conditions used for the preparation of the lipide-free filtrates. Such a criterion was of critical importance since quantitative precipitation of lipides from aqueous emulsions could only be accomplished at slightly acidic reaction. All pertinent observations on plasmalogens demonstrate the unusually high lability of their aldehydogenic lipide groups toward acid reaction.

Neither the Schiff reaction nor other aldehyde tests such as condensation with phenylhydrazine derivatives could be directly used as tests for the liberation of aldehydes since these reactions require acidities resulting in rapid cleavage of the bound aldehydogenic groups in the plasmalogens. The only test which was available for this purpose was the "aldehyde disappearance reaction" described by Schmidt et al.[23] in 1957. This test is based on the observation that free lipide aldehydes are converted quantitatively to Schiff-negative compounds (perhaps by polymerization) when incubated in aqueous emulsions in phosphate buffer at pH 8.5, whereas plasmalogens do not change their behavior toward Schiff's reagent under these conditions.

It was found that incubation of plasmalogen-containing lipide emulsions at pH 5.5 for 5 hours, followed by incubation under the conditions of the aldehyde disappearance reaction had no influence on the extinction observed in the Schiff test. The same result was obtained when the incubation at pH 5.5 was carried out in a half-saturated solution of ammonium sulfate.

III. *Hydrolysis of the aldehydogenic Group of the Plasmalogens.*—The basic observations regarding the behavior of the plasmalogens toward hydrolyzing agents were made many years ago by *Feulgen* and his collaborators.[19] They may be summarized by stating that the aldehydogenic groups of the plasmalogens are very labile toward acids and toward mercury (2) chloride, but very stable toward alkali. Owing to the rapid reaction of plasmalogens with fuchsin-sulfurous acid, Feulgen's observations regarding the behavior of plasmalogens toward hydrolyzing agents were of a qualitative nature and left many problems unanswered such as the kinetics of hydrolysis, the question as to whether the effect of mercury (2) chloride was caused by the mercury-halide complex or by mercury (2) ions †; the question as to whether mercury (2) chloride itself catalyzed the cleavage or whether it increased the acid-lability of the plasmalogens, and other problems which will be discussed in the following sections.

A. Hydrolysis of Aldehydogenic Lipide Groups by 0.25 N Hydrochloric Acid: For the purpose of this investigation, the interest in the behavior of aldehydogenic lipide groups toward acids was limited to the question as to whether complete cleavage of these groups was achieved under conditions of acidity and reaction time which resembled those required for maximal color development in the Feulgen test at room tempera-

† Feulgen, Boguth, and Andresen[24] reported that addition of sodium chloride to the reaction mixture of the Feulgen test with serum lipides inhibited the development of the color reaction. They attributed this effect to the formation of mercury (2) chloride complexes.

TABLE 3.—*Partition of Brain Cephalin into Ether Phospholipides and Plasmalogen*

Treatment of Cephalin Emulsion	Total Phosphorus Mg	Water-soluble Phosphorus Mg	Per-cent of Total P	Fuelgen Extinction E (555 mμ) in n-Amyl Alcohol per M Mole/Ml of Mercury Labile
None	0.180	—		2.2×104
0.066 N mercuric acetate, 1 hr. 25°	0.180	0	0	—
1 N sodium hydroxide 18 hours, 37°	0.180	0.142	78.9	—
Incubation in 0.055N mercuric acetate, 1 hr. followed by incubation with N NaOH, 18 hrs, 37°	0.180	0.182	101.1	—
Incubation with 0.25 N HCl, 30 min.	0.180	0	0	—
Incubation with 0.25 N HCl for 2 hrs. 25°, followed by incubation in N NaOH 18 hours, 37°	0.180	0.168	93.3	—
Incubation with N NaOH for 18 hrs., 37° followed by incubation with 0.25N HCl 3 hrs, room temp.	0.180	0.170	94.4	—

ture in absence of mercury salts. Extension of the hydrolysis time or selection of more drastic temperature or acidity conditions would not have been feasible for tissue analysis, owing to the limited stability of sphingophospholipides at acid reaction.

The experiments were carried out on emulsions of brain cephalin (total cephalin fraction of Folch [16]) which was free of sphingomyelin as judged by the absence of choline from the aqueous phase of a hydrolysate obtained by heating a sample for two hours in a boiling water bath with 0.25 M aqueous barium hydroxide.

A portion of an aqueous cephalin emulsion was shaken in 0.25 N aqueous hydrochloric acid (final concentration) for 3 hours at 25°. The neutralized reaction mixture was subsequently saponified in N sodium hydroxide for 20 hours at 37°. A second equal portion of the cephalin emulsion was incubated in the reverse order with alkali and then with acid under analogous conditions. (The volume of the acid hydrolysis mixture of the second (saponified) sample was adjusted to the twenty-fold volume of the alkaline digest for the purpose of minimizing the buffer effect of the sodium chloride formed during the neutralization of the alkaline digest.) The amounts of water-soluble phosphorus formed during the incubations of the two samples are recorded in the last two lines of Table 3. They accounted respectively for 93.3 and 94.4 per cent of the total phosphorus of the cephalin emulsion. Since 78.9 per cent of the cephalin consisted of ester phospholipides these figures indicate that only 68.2 and 73.5 per cent of the plasmalogen phosphorus of the respective cephalin samples had been converted to water-soluble phosphorus compounds under the conditions described above. When the action of 0.25 N hydrochloric acid on cephalin emulsions was assayed by means of the aldehyde disappearance reaction, degrees of hydrolysis ranging between 70 and 80 per cent were observed.

The incomplete conversion of the plasmalogen phosphorus to water-soluble phosphorus by subsequent treatment of cephalin with acid and alkali was unexpected in view of the results of experiments in which the decrease of genuine and of alkali-treated plasmalogen during incubation with hydrochloric acid was assayed by the aldehyde-disappearance reaction. Representative results of such experiments are recorded in Table 4. The rates of decrease of plasmalogens dur-

TABLE 4.—*Time Course of Hydrolysis of Non-Saponified and Saponified Plasmalogen with 0.2N Hydrochloric Acid*

Time of Acid Hydrolysis, Minutes	Per Cent Hydrolysis	
	Non-Saponified Plasmalogens	Saponified Plasmalogens
5	34	31
10	40	42
15	45	49
30	57.5	65
60	74	83
120	90	98
190	97	—

ing incubation of aqueous emulsions of non-saponified and saponified brain lipides were similar, and the plasmalogen concentrations fell to values below 5 per cent of the initial concentration after 3 hours' incubation at room temperature.

Possibly, the variability of the extent of the hydrolysis of the aldehydogenic lipide groups with acids might be caused by differences of the degrees of dispersion of lipide emulsions of different origin. It was noted that emulsions of cephalin precipitated in flocculent form immediately after acidification whereas emulsions of the total brain lipides remained in fine dispersion during the incubation with hydrochloric acid. Feugen et al.[25] already pointed out the considerable influence of the degree of dispersion on the extent of the Schiff reaction of lipide emulsions.

It is also possible that a deficit of the yield of water-soluble phosphorus compound might be produced by a secondary recombination of liberated lipide aldehydes with phosphoric acid esters of ethanolamine or serine. Such an assumption would be compatible with the observations of Thiele [26] who reported that the Feulgen reaction was inhibited by primary amines, presumably because of the formation of Schiff bases.

Regardless of the theoretical interpretations of these observations, the results of the experiments show clearly that treatment of lipide mixtures with acid is not a suitable step for the selective and quantitative degradation of plasmalogen to water-soluble phosphorus compounds.

B. Hydrolysis of Aldehydogenic Lipide Groups by Mercury (2) Compounds: The course of the catalytic hydrolysis of the aldehydogenic lipide groups with mercury (2) salts was studied with the purpose of exploring the suitability of mercury (2) salts as catalysts for the quantitative and specific hydrolysis of plasmalogens. The progress of hydrolysis was followed by determinations of the amounts of water-soluble phosphorus compounds formed during saponification of the samples which had been incubated with mercury (2) salts. The subsequent saponifications were carried out in N sodium hydroxide at 37° for 20 hours.

1. Mechanism of hydrolysis of plasmalogens by mercury (2) salts. When plasmalogen-containing aqueous emulsions of brain cephalin were mixed either with mercury (2) acetate or with mercury (2) chloride (final concentration of the mercury (2) compounds 0.035 M), and subsequently incubated with N sodium hydroxide at 37° for 20 hours, the lipide phosphorus was completely converted to water-soluble phosphorus compounds (Table 3, line 4). The cleavage of the aldehydogenic lipide groups of non-saponified plasmalogens by the mercury (2) salts was practically instantaneous because the extent of cleavage was not appreciably influenced by the time interval elapsing between the addition of the mercury (2) salts and that of the sodium hydroxide.‡ (The catalytic action of the mercury (2) compounds was stopped by the addition of excess alkali since no hydrolysis of the aldehydogenic lipide groups occurred when mercury (2) salts were added to an emulsion of cephalin in N sodium hydroxide.) Two conclusions may be drawn from this experiment: 1. The catalytic hydrolysis of aldehydogenic lipide groups is effected by mercury (2) *ions* rather than by its halide complexes. 2. The action of mercury (2) ions does not depend on the acidification of the reaction mixture produced in the Feulgen test by the presence of sulfurous acid.

2. Action of mercury (2) acetate on alkali-treated plasmalogens (acetal phospholipides). The aldehydogenic lipide groups of alkali-treated plasmalogens are hydrolyzed more slowly by mercury salts than those of the genuine plasmalogens. Whereas those of the latter are completely hydrolyzed within fractions of a minute, those of the former approach completion after a two hour incubation with mercury (2) acetate.

The time course of the mercury-catalyzed hydrolysis of alkali-treated plasmalogen is

‡ The instantaneous cleavage of genuine plasmalogens by mercury (2) chloride was already observed by Feulgen et at.[27]

illustrated in Figure 2, curve *B*. The shaded part of the bar shows the amounts of water-soluble phosphorus released during hydrolysis with 0.25 N hydrochloric acid of a portion of the same sample of alkali-treated plasmalogen. A comparison between curve *B* and the height of the bar at the top of the shaded area demonstrates that the amounts of water-soluble phosphorus liberated during incubation with hydrochloric acid were appreciably smaller than those formed during a 10 hour incubation with mercuric acetate, and that the curve of the mercury hydrolysis did not reach a plateau after 4 hours. Hydrolysis of alkali-saponified plasmalogens either with mercury ions or with acid fails, thus, to offer a well defined end point and progresses too slowly for application in tissue analysis.

3. Specificity of action of mercury (2) ions on aldehydogenic lipide groups. No water-soluble phosphorus compounds are formed when emulsions of cephalin or of crude lipides of brain or spinal cord are shaken with mercury (2) salts for one hour without a subsequent saponification with N sodium hydroxide (Table 3, line 1). This confirms the earlier conclusion of Schmidt et al.[9,10] that plasmalogens contain in addition to the aldehydogenic group, a second lipide group which is not hydrolyzed in the presence of mercury (2) ions. Since Klenk et al.[13] and Rapport [1] have shown that this second lipide group is a fatty acid group, it must be concluded that the fatty acid ester group of plasmalogens is resistant to mercury (2) salts. It should be emphasized, however,

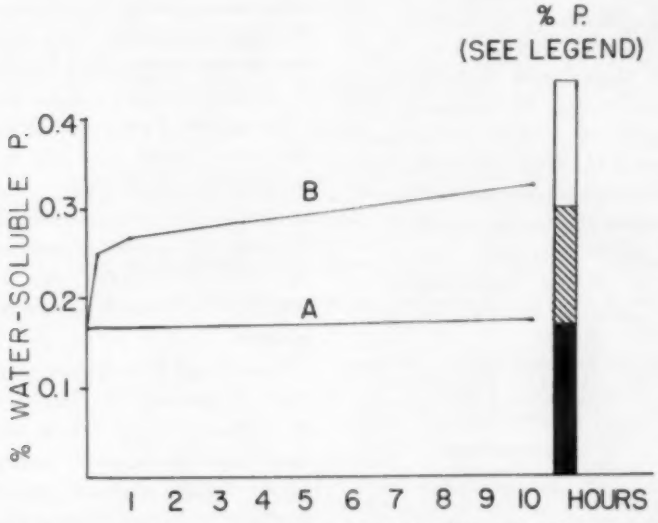

Fig. 2.—Time course of hydrolysis of alkali-treated plasmalogens by mercury (2) chloride. Material: The total lipides of 4.0 gms of beef spinal cord were saponified in 40 mls of aqueous N sodium hydroxide for 20 hours, the emulsion was neutralized to pH 5.5 by addition of glacial acetic acid, and diluted with water to a volume of 50 mls. (Emulsion S) Curve A: 10 mls of S shaken with 2 mls of water at 25°. Curve B: 25 mls of S shaken with 5 mls of a 5 per cent aqueous solution of mercury (2) chloride at 25°. The ordinates of curves A and B represent the amounts of total water-soluble phosphorus obtained at the time intervals indicated after precipitation of the lipides with ammonium sulfate at pH 5.5. Bar graph: Amounts of water soluble phosphorus formed by shaking of *alkali*-treated plasmalogens with 0.25 N hydrochloric acid at pH 0.8 at 25°. (Shaded part of bar.) Preparation of saponified lipide emulsion as described above except that neutralization was carried out by titration with N hydrochloric acid under cooling and subsequent adjustment of volume to 100 mls; 10 mls of neutralized emulsion were shaken with 40 mls of 0.31 N hydrochloric acid for 2.5 hours at 25°. The water-soluble phosphorus was determined in the clear filtrate. The total height of the bar represents the amount of total lipide phosphorus, the black part of the bar that of the ester phospholipide phosphorus. All phosphorus values are expressed as grams per 100 grams of fresh tissue.

that the specificity of the catalytic action of mercury (2) salts on the hydrolysis of aldehydogenic lipide groups is only valid for aqueous lipide emulsions for the temperature conditions defined above. Tattrie and McArthur [28] demonstrated that refluxing of ethanolic solutions of ester lecithin in presence of mercury (2) chloride results in such extensive cleavage of the fatty acid groups that a very convenient procedure for the preparation of L-glycerylphosphorylcholine could be developed on the basis of this behavior. Sphingomyelin does not yield measurable amounts of water-soluble phosphorus compounds after incubation with mercury (2) acetate and subsequent saponification with N sodium hydroxide under the conditions described.

The catalytic cleavage of the aldehydogenic groups of the plasmalogens and of the acetal phospholipides by mercury (2) ions is not a reaction of acetals. It was found that the slow liberation of lipide aldehydes which occurred during the incubation with Feulgen reagent of aqueous emulsions of dimethyl acetals of lipide aldehydes (prepared according to Leupold and Buettner [29]) was not enhanced by the addition of mercury (2) salts.

4. Influence of the concentration of mercury (2) ions on the yield of water-soluble phosphorus compounds from plasmalogens. The yields of water-soluble phosphorus compounds were studied at mercury concentrations ranging from 0.0013 to 0.078 molarity. Table 5 shows the results of this experiment.

TABLE 5.—*Influence of Concentration of Mercury (2) Ions on Formation of Water-Soluble Phosphorus Compounds from Plasmalogen*

Mercury (2) Acetate MMoles/Ml	Water-Soluble Phosphorus Compounds Formed Mgm P per Sample
0.00	1.030
0.0026	1.260
0.013	1.280
0.063	1.276
0.126	1.280

Lipide emulsion: Aqueous emulsion of brain cephalin containing 1.280 mgm total phosphorus and 1.03 mgm ester phospholipide phosphorus in 10 mls. 10 ml portions were mixed with 5 mls of the respective solutions of mercury (2) acetate and shaken at room temperature for 60 minutes. The emulsions were then saponified in N sodium hydroxide, and the water-soluble phosphorus was determined in the ashed solutions of the lipide-free filtrates.

The data show that the yields remained constant between mercury concentrations ranging between 0.039 and 0.078 molarity, and that they increased only by 7.7 per cent (related to plasmalogen P) over the range of 0.0013 and 0.039 molarity. It should be pointed out that the lowest mercury concentrations studied were at the order of magnitude of the concentration of plasmalogen present during the reaction. For this reason, the data do not permit to decide the question as to whether a small amount of mercury (2) ions catalyzes the cleavage of a large amount of plasmalogens, or whether the hydrolysis results from a stoichiometric reaction between mercury (2) ions and plasmalogen.

The action of mercury (2) ions on saponified plasmalogens was only studied in the presence of a large excess of mercury (2) acetate because a considerable part of added mercury (2) ions is immediately bound in the form of insoluble salts by the large quantity of fatty acids present in the reaction mixture.

Table 6 and Table 7 show that the catalytic effect of mercury (2) ions on the cleavage of aldehydogenic lipide is highly specific for these ions. None of a number of other metals studied, produced more than a slight degree of hydrolysis of genuine or saponified plasmalogens. The actual differences between the effects of mercury and those of the other metals are certainly much larger than those indicated by the data of Table 6 in view of the fact that in this experiment, the samples of plasmalogen-containing emulsions were shaken for one hour in presence of the respective metal salts whereas the effect of mercury (2) ions is pratically instantaneous.

TABLE 6.—*Specificity of Catalytic Effect of Mercury (2) Ions on Hydrolysis of Non-Saponified Plasmalogens*

Metal Salts	None	Hg	Cd	Mn	Fe	Cu	Co	Zn
Water-soluble P formed (mg per 100 gm of fresh tissue)	0.167	333	0.168	0.192	0.160	0.163	0.165	0.180

Material: 5 mls of an aqueous emulsion of the non-saponified lipides of beef spinal cord. The samples were mixed with portions of 0.8 mls of the respective solutions of the metal salts (final concentration 0.032 M). After shaking for 1 hour at room temperature the samples were saponified in N sodium hydroxide for 20 hours. The amounts of the water-soluble phosphorus were determined in each sample after precipitation of the lipides with ammonium sulfate at pH 5.5.

TABLE 7.—*Specificity of Catalytic Effect of Mercury (2) Ions on Hydrolysis of Saponified Plasmalogens*

Metal salt	None	Hg	Cd	Ag	Mn	Fe
Mg Water-Soluble P Formed	0.004	0.120	0.012	0.005	0.004	0.003

Material: 5 ml of an aqueous saponified emulsion of the lipides of 100 mg of beef spinal cord (saponification in N sodium hydroxide at 37° for 18 hours). The saponified emulsion was brought to pH 5.5 by the addition of glacial acetic acid; 5 ml-portions were mixed with the respective solutions of the metal salts and shaken at room temperature for 3 hours. The final concentration of the metal salts was 0.004 M in each sample. The lipides were then removed as described in the section on methods, and the total phosphorus was determined in the clear filtrates.

Distribution of Phospholipide Fractions in Some Tissues

In Tables 8 and 9, representative data of the distribution of the three phospholipide fractions are reported for some tissues. The last two columns contain the extinctions of the tissue lipides found in the Feulgen test at 555 mμ in 20 ml of n-amyl alcohol. The values of the last two columns represent respectively the extinctions of the lipides of 100 gms of fresh tissues in 20 ml of n-amyl alcohol, and those per mole of mercury labile lipide phosphorus in a liter of n-amyl

TABLE 8.—*Distribution of Phospholipides in Some Tissues*

Species	Organ	Total Phospholipides	Ester Phospholipides	Plasmalogens	Sphingo and Ester Phospholipides	Feulgen Extinction of Total Lipides E (555 mμ) in n-Amyl Alcohol	
		Mg P per 100 Gm Moist Tissue (Per Cent P of Total Lipide P)				per 100 Gm Tissue (in 20 Ml Amyl Alcohol)	per M Mole/Ml of Mercury-Labile Lipide-P
Beef	Brain	208	132 (63)	53 (26)	23 (11)	1400	1.7×10^{4}
Beef	Spinal Cord	430	183 (42.5)	144 (33.3)	101 (23.4)	4100	1.8×10^{4}
Beef	Cerebellum	204	125 (61.0)	64 (31.5)	15 (7.4)	2000	2.6×10^{4}
Beef	Heart	94	63.5 (67.5)	25.8 (27.4)	4.7 (5.0)	1050	2.5×10^{4}
Rat	Heart	91	68.5 (75.2)	19.0 (20.9)	3.5 (3.9)	—	—
Dog	Heart	66.5	39.0 (58.7)	20.4 (30.1)	7.7 (10.7)	600	2.4×10^{4}
Rat	Skeletal muscle	38.2	32.4 (85.0)	3.8 (10.0)	2.0 (5.0)	—	—
Dog	Skeletal muscle	32.4	25.2 (78.0)	6.2 (18.9)	0.7 (2.1)	—	—
Rabbit	Skeletal muscle	19.3	15.0 (77.7)	2.8 (14.6)	1.5 (7.7)	—	—
Rat	Liver	91.0	88.0 (97.0)	1.0 (1.0)	2.0 (2.0)	—	—
Beef	Spleen	80.0	59.0 (73.8)	12.5 (15.6)	10.6 (10.6)	410	2.0×10^{4}

TABLE 9.—*Distribution of Phospholipides in Some Tissues*

Species	Organ	Total Phospholipides	Ester Phospholipides	Plasmalogens	Sphingo and Ester Phospholipides	Feulgen Extinction of Total Lipides E (555 Mμ) in N-Amyl Alcohol	
		Mg P per 100 Gm Moist Tissue (Per Cent P of Total Lipide P)				per 100 Gm Tissue (in 20 Ml Amyl Alcohol)	per M Mole/Ml of Mercury-Labile Lipide-P
Beef	Kidney	102	80.0 (78.4)	8.2 (8.0)	13.8 (13.6)	260	1.9×10^{4}
Beef	Pancreas	123	105.0 (84.7)	10.2 (8.3)	9.0 (7.3)	—	—
Beef	Testes	68.4	52.2 (76.3)	9.0 (13.1)	7.2 (10.6)	200	2.0×10^{4}
Ram	Testes	51.4	35.8 (68.7)	7.2 (15.0)	8.4 (16.3)	280	2.5×10^{4}
Ram	Epididymis	52.5	38.4 (74.0)	7.4 (13.9)	6.5 (12.1)	300	2.4×10^{4}
Beef	Adrenals	150	122.0 (81.3)	17.7 (11.8)	11.3 (7.0)	560	2.0×10^{4}
Beef	Thyroid	27	18.6 (68.9)	4.6 (17.0)	3.7 (14.1)	—	—
Fowl	Egg Yolk	430	422.0 (89.1)	negligible	8.0 (1.9)	—	—

alcohol. The data reveal the essential parallelism between the concentrations of the mercury-labile lipide phosphorus and the Feulgen extinction of the lipides per 100 gms of tissue.

The Feulgen extinctions per mole per liter of mercury-labile lipide phosphorus ranged between 16,000 and 18,800 for the lipides of brain and spinal cord, and averaged about 20,000 for several other tissues as well as for cephalin samples isolated from brain and spinal cord. The interpretation of these differences will remain conjectural until it will be possible to determine the molar Feulgen extinctions of pure genuine plasmalogens. Since these phospholipides cannot be isolated at present in pure form, only model compounds are available for reference, such as crystalline, saponified plasmalogens or free lipide aldehydes. A crystallized sample of saponified plasmalogen from brain which had been prepared according to Thannhauser, Boncoddo and Schmidt [12] gave a molar Feulgen extinction of 17,000 whereas M. M. Rapport found for crystallized, saponified plasmalogen from beef skeletal muscle the value of 23,000. On the basis of the data of Feulgen and Gruenberg,[19] Feulgen, Boguth, and Andresen [24] and of Leupold and Buettner, the molar Feulgen extinction of free lipide aldehydes at 555 mμ in amyl alcohol is close to 30,000.

The need for pure plasmalogens as reference materials in the quantitative evaluation of the Feulgen extinctions is particularly indicated by the observations of M. M. Rapport et al.[1] who postulated the structure of vinyl ethers rather than that of acetals for the genuine compounds. According to this concept, the lipide aldehydes which appear after cleavage of plasmalogens are secondary products formed by hydration of liberated vinyl alcohols. The yield of lipide aldehydes as products of this hydration is unknown at present.

Finally, Ehrlich, Taylor, and Waelsch [31] found that the color development in the Feulgen reaction with crude lipide emulsions was strongly influenzed by the presence of surface-active substances.

The fact that the molar Feulgen extinctions of cephalin samples which were isolated from brain (Table 3) or from spinal cord were found to be approximately 15 per cent higher than those calculated from the data obtained with the total lipides of the corresponding tissues suggests the possible presence of inhibitors of the

Feulgen reaction in crude tissue lipide mixtures.

Thiele[26] reported observations demonstrating appreciable degrees of inhibition of the Feulgen reaction by primary amines, perhaps by formation of Schiff bases with the aldehyde groups. In view of the comparably high molar extinction given by purified cephalin fractions in the Feulgen reaction, it is unlikely, however, that the low molar Feulgen extinctions observed with emulsions of the total lipides of brain and spinal cord were caused by the presence of amino groups.

Comparison Between the Rates of Diffusion of Plasmalogen and of Lecithin

The plasmalogen fraction accompanies the cephalin fraction throughout the various steps involved in the preparative fractionation of brain lipides. This behavior is so characteristic that it has been impossible so far to isolate cephalins which do not contain considerable quantities of plasmalogens, except from tissues in which plasmalogens occur only in negligible quantities.[32] This raises the question as to whether or not a part of the plasmalogen of brain might exist as a compound with cephalin. The analytical implications of this possibility are of interest since the existence of compounds of plasmalogens with other phospholipides would explain the somewhat low ratio between Feulgen extinction and mercury-labile lipide phosphorus which was reported in Table 8 as characteristic for the plasmalogens of nervous tissue.

A compound between plasmalogen and cephalin would be expected to have at least twice the molecular weight of that of lecithin, and to have an appreciably smaller diffusion rate than lecithin. The diffusion rates of cholesterol, of egg lecithin (prepared according to Hanahan, and Jayko[17]), and of plasmalogen were determined in a Northrop cell at 0.005 M concentration of the lipides with 95 per cent ethanol as solvent. The determinations were carried out at 26.5°. An alcoholic extract of acetone-dried beef spinal cord was used for the diffusion experiments with plasmalogen. The quantitative Feulgen test was used for the plasmalogen determinations. The amounts diffusing per hour were respectively 0.135, 0.092, and 0.092 per cent of the original solutions of cholesterol, lecithin, and plasmalogen. In view of the identity of the diffusion rates, of lecithin and plasmalogen, it seems unlikely that appreciable amounts of plasmalogen occur in the form of compounds with other lipides of similar molecular weights such as cephalin.

Comment

1. *Structure of Plasmalogens.*—All studies concerning the structure of plasmalogens must be based at present on indirect evidence since only their degradation products, but not the genuine aldehydogenic phospholipides, can be isolated in reasonably pure form. The few guiding reactions which permit the correlation of the degradation products with the genuine aldehydogenic lipides are still based on the early pioneering investigations of Feulgen and his collaborators.[27] These are the lability of the bond between the aldehydogenic group and the other moiety of the plasmalogen molecule toward acids and toward mercury (2) chloride and its resistence toward cleavage by alkali. (This resistance does not imply preservation of the original structure of the bond.) The application of these guiding reactions to the analysis of plasmalogen-containing lipide mixtures contributed, in historical sequence, the following facts pertinent to the present concept of the structure of plasmalogens ‖: 1) The isolation of lipide aldehydes after cleavage of muscle phospholipides by mercury (2) chloride (Feulgen et al.,[27] 1929); 2) the isolation of crystallized lysophosphatidal ethanolamine from saponified lipides of muscle (Feulgen et al.[11] 1939) and brain (Thannhauser et al.,[12]

‖ Some important structural aspects which are not directly pertinent to the problems of the present investigations, such as the nature of the basic groups, have been omitted from this brief survey.

1951); 3) the isolation of the phosphoric acid esters of batyl and chimyl alcohol from hydrogenated brain cephalin after hydrolysis and their recognition as derivatives of lysophosphatidals (Klenk and Debuch,[13] 1954; 4) the detection of the different nature of the phosphorus-containing degradation products obtained by acid- or mercury-catalyzed hydrolysis from crystallized lysophosphatidals on the one hand, and from genuine plasmalogens on the other hand (Schmidt et al.,[9,10] 1951, 1953). On the basis of these differences, Schmidt et al.[9,10] postulated that the lysophosphatidals were secondary degradation products which originated from the genuine plasmalogens by the removal of an alkali-labile lipide group during the saponification procedure employed for their isolation. 5) The isolation of lysophosphatides from plasmalogen-containing phospholipide mixtures after mild acid hydrolysis (Klenk and Debuch,[33] 1955; Debuch,[14] 1956; Rapport and Alonzo,[34] 1955). These observations demonstrated that the alkali-labile lipide group postulated by Schmidt et al.[9,10] as a constituent group of genuine plasmalogens must be a fatty acid group. 6) The addition of iodine by crystallized lysophosphatidal, suggesting the structure of a vinyl ether for the aldehydogenic group of plasmalogen (Rapport et al.,[1] 1957).

The studies by Schmidt et al.[9,10] resulted in the first observations which demonstrated the necessity for a revision of Feulgen's original concept of the chemical structure of plasmalogen.[11] Schmidt et al.[9] postulated already in 1951—several years before the publication of pertinent reports from other laboratories—the presence of a second lipide group in the plasmalogen molecule, in addition to the aldehydogenic lipide group. The nature of the second lipide group was subsequently identified as that of a fatty acid group by Klenk and Debuch,[33] Debuch,[14] and Rapport and Alonzo.[34]

The evidence presented in this paper on the basis of the new hydrolysis technique supports the validity of the definition of the plasmalogens as fatty acid esters of lysophosphatidals for the total plasmalogen fraction of all tissues analyzed so far. This justifies the use of the mercury-labile lipide phosphorus as a measure for the plasmalogen content of tissues.

Apart from the quantitative extent and the rapidity of the action of mercury salts on genuine plasmalogens, they offer the advantage of far greater specificity of the catalytic effect in comparison to acid hydrolysis.

2. *The Problem of "Cephalin B."*—According to observations of Brante,[5] Edgar,[6-8] and Schmidt et al., the application of the earlier method of Schmidt et al.[4] for the partition of tissue phospholipides to crude brain cephalin which did not contain appreciable quantities of sphingomyelin, yielded measurable amounts of an alkali-resistant phospholipide fraction which was designated by Brante[5] as cephalin B. Since this fraction was never observed when the modified procedure described in this paper was used it is very likely that the appearance of cephalin B was caused by incomplete hydrolysis of saponified plasmalogens.

3. *Determination of Sphingomyelin.*—The sum of ether phospholipides and of sphingomyelin may be determined by difference between the total lipide phosphorus, and between the sum of ester phospholipides and of plasmalogens. Since the sphingomyelin fraction accounts for a relatively small part of the total lipide phosphorus, completeness of the conversion of the ester phospholipide and of the plasmalogen phosphorus to water-soluble phosphorus compounds is obviously of critical importance for the determination of the sphingomyelin fraction. This requirement is only met by the hydrolysis procedure described in this paper. When the separate determination of ester phospholipides and of plasmalogens is not desired, a simplified technique for the determination of sphingomyelin may be applied. This modification will be described in another paper in the near future. While the comparison between the sphingomyelin values recorded in Tables 8 and 9 of this paper and those listed in an earlier investigation by Schmidt et al.

does not reveal major discrepancies it must be pointed out that the application of the earlier method to tissues with high plasmalogen concentrations such as nervous tissue, skeletal muscle and heart might yield erroneously high values for the sphingophospholipide fraction because of incomplete conversion of plasmalogen to water-soluble phosphorus. Mallov, McKibbin, and Robb[36] reported that the earlier hydrolysis method of Schmidt et al.[4] when applied to beef heart, yielded higher sphingomyelin figures than those calculated from the results of determinations of total sphingosine. In the light of the observations described in this paper, these discrepancies were probably caused by incomplete hydrolysis of aldehydogenic phospholipides which occur abundantly in beef heart. The sphingomyelin figures of beef heart which were obtained with the new hydrolysis method (Table 8 of this paper) are compatible with the total sphingosine concentrations found by Mallov et al.[36] in this tissue.

Summary

1. A procedure for the quantitative partition of tissue phospholipides into ester phospholipides, plasmalogens, and the sphingomyelin fraction (sum of ether and sphingophospholipides) has been described. The procedure is based on phosphorus determinations.

2. Comparative studies of the behavior of the aldehydogenic lipide groups of genuine and saponified plasmalogens toward dilute hydrochloric acid and toward mercury (2) salts have been carried out with the result that the cleavage of genuine plasmalogens by mercury (2) salts is the only suitable method for the quantitative partition of lipide mixtures on the basis of phosphorus determinations.

3. The bearing of the analytical results on the concept of the structure of plasmalogens has been discussed.

4. Representative values for the distribution of the individual phospholipides in some tissues have been reported.

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Introductory Remarks, Afternoon Session

W. M. SPERRY, Ph.D.

It is a distinct honor for me to serve as the Chairman of this program which includes several of the world's leading biochemists, particularly in the lipide field. I have a rule which I usually follow when I serve as Chairman of a program with several speakers; that rule is that I utter no words of praise concerning a speaker when I introduce him and no words of praise of the paper after he is finished. I adopted that rule long ago because I observed that most speakers are human beings, and, being such, they vary widely among themselves in the excellence of the research they have carried out and in their ability to present it. In other words to put it bluntly, some papers are better than others.

I have taken this moment in order to explain, for the benefit of the other speakers, that I am going to violate my rule in introducing the first speaker. I am sure the other speakers on the program will not resent this exception which I intend to make because our first speaker would merit some special words of praise if for no other reason than that he is our distinguished guest from abroad. But even if Ernst Klenk had been born in this country and had carried out his work here, I think I still would violate my rule because he, more than any other man, has provided us with knowledge, in fact about the only knowledge we have, concerning the biochemical lesion which underlies Tay-Sachs' disease.

In 1935, I believe it was, he first obtained evidence for a new lipide in the brain which he called substance X. In 1940, he found that this substance X, which he later called ganglioside, is present in greatly increased amounts in the brains of Tay-Sachs patients, and, therefore, the underlying lesion is an overproduction or underutilization of these gangliosides. As far as I am aware nothing is known of the mechanism. I am hoping that he is going to tell us something about it today.

In making that discovery he made another equally important, perhaps more important contribution. I think possibly for the first time, he provided a means for the differential diagnosis between two diseases on biochemical grounds. He had previously shown that in Niemann-Pick's disease there are very large accumulations of sphingomyelin in the liver, spleen, and other tissues throughout the body. This does not occur at all in Tay-Sachs' disease, whereas the accumulation of gangliosides in such large quantities in the brain does not occur in Niemann-Pick's disease. They are obviously entirely distinct diseases, regardless of their physical similarities.

And in that same series of investigations he made a third major discovery which in its broad aspects is the most important of all. He discovered neuraminic acid as a constituent of these gangliosides, and just in the past two or three years it has become known, to a large extent through Dr. Klenk's own work, that this neuraminic acid, or its acetyl derivative, sialic acid, is widely distributed in nature and apparently is a very important substance indeed.

Dr. Klenk has made many other important discoveries, but I can't take time to tell you about them. I thought that the occasion did justify the time I have taken to summarize these findings which relate directly to the subject of this conference.

I will now conclude this introduction by a very brief resume of his career which I am going to read from his curriculum vitae. He received his Ph.D. degree in 1923. He remained at the University of Tübingen until 1936 and worked there with Thier-

felder and Knoop, both honored names in the field of lipide biochemistry. He was appointed as Professor in 1932.

In 1936 he went to the University of Cologne, where he still is Professor of Physiological Chemistry and Director of the Institute for Physiological Chemistry. In 1947-48 he was Dean of the Faculty of Medicine at the University of Cologne. He was made an Honorary Doctor of Medicine by the Faculty of Medicine at the University of Cologne in 1948 and he was given the Norman Medal of the Deutsche Gesellshaft für Fettwissenschaft in 1953.

It is a great pleasure for me to introduce Professor Klenk, who will talk to you on the "Gangliosides" which he discovered.

New York State Psychiatric Institute, New York.

On Gangliosides

Prof. Dr. E. KLENK

One day almost 25 years ago, when I was still a Privatdozent at Tübingen, I had unexpected visitors from Basel. The pathologist and a physician for children's diseases from that town had come to ask me to investigate some organs of an infant that died with a lipid storage disease. It was a case of Niemann-Pick's disease. My visitors only wanted me to find out which lipid was accumulated in this case, a task which was not difficult and which could be solved rather quickly.

The accumulated lipid was sphingomyelin, which together with the cerebrosides belongs to the sphingolipids. In normal spleen and liver the sphingomyelin is to be found only in traces, but in Niemann-Pick's disease it occurs in amounts of about 20% of the dry weight of the organs.

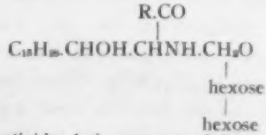
Almost simultaneously with the case of Niemann-Pick's disease a case of amaurotic idiocy of the Tay-Sachs type was admitted to the children's hospital of Basel. In this case, in contrast to Niemann-Pick's disease, the lipid storage is known to be limited to the nervous tissue. Based upon the histological findings, most of our pathologists were at that time of the opinion that the accumulated substance was identical with the lipid found in Niemann-Pick's disease, i. e., sphingomyelin. The chemical investigation, however, of the Tay-Sachs brain yielded quite a different result. Sphingomyelin was almost completely absent and the main lipid found instead was a sugar containing substance differing from all known brain lipids. The substance was completely soluble in water and formed an extremely large amount of humin substances when heated with dilute acids. Bials' sugar reaction was produced by the substance, with a red-violet colour instead of the green colour given by the cerebrosides. The substance belonged to a new group of hitherto unknown glycolipids which I called gangliosides. They are also normal brain constituents and are predominantly found in the gray matter of the brain containing the ganglionic cells, whereas the cerebrosides are known to be typical constituents of the myelin sheath.

The gangliosides are sphingolipids like the cerebrosides and the sphingomyelins, but they are distinguished from them by their distinctly acid properties. Their characteristic component is neuraminic acid, a polyoxyamino acid, which is closely related to the sugars. After the cleavage of the gangliosides with methanolic hydrochloric acid, neuraminic acid could be obtained as the well crystallizing methoxy derivative. The compound is extraordinarily unstable toward acid. When heated with n/10 hydrochloric acid or sulfuric acid it completely decomposes with the formation of black humin substances so that the free neuraminic acid itself could not be isolated. The acid imparts the previously mentioned Bial reaction with a red violet colour. Since this reaction is especially sensitive it is possible to determine amounts of 10-20γ colorimetrically without difficulties. The colour reaction with Ehrlich's aldehyde reagent, which is characteristic of acetylated aminosugars, is also given by neuraminic acid, with the difference, however, that the aminosugars give this reaction only after alkali pretreatment while the neuraminic acid reacts without such a pretreatment. The elementary composition of the methoxy neuraminic acid corresponds to the formula $C_{10}H_{19}NO_8$ or $C_{11}H_{21}NO_9$. Since both the C-H and N values are too close together, it is impossible to choose one or the other

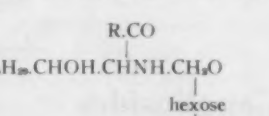
From the Institute for Physiological Chemistry of the University, Cologne.

formula only upon the results of the elementary analysis. Until recently I have preferred the formula $C_{11}H_{21}NO_9$. Today the formula $C_{10}H_{19}NO_8$ must be considered to be correct so that the formula of the neuraminic acid itself would be $C_9H_{17}NO_8$. It is apparently a condensation product of pyruvic acid and hexosamine as it has exactly been predicted by Gottschalk.

Glycolipids, which are closely related to the gangliosides, have also been found in other organs. In connection with the lipid storage diseases, we were especially interested in the glycolopids of the spleen and of the red blood cells. In Gaucher's disease, which has now been recognized as the third form of the lipid storage diseases, the cerebrosides are accumulated within the reticular system and especially in the spleen. We have carried out these investigations because we assumed a close connection between the ganglioside and cerebroside metabolism. The main component of the glycolipid fraction obtained from spleen of beef on this occasion was a substance very similar to the brain gangliosides and containing, like the brain gangliosides, more than 20% of neuraminic acid. Finally Yamakawa has isolated a ganglioside-like lipid from stroma of horse erythrocytes, while we at the same time investigated the glycolipids of human erythrocytes and of the stroma of beef erythrocytes finding only neuraminic acid-free glycolipids. When re-investigating the glycolipids of horse erythrocytes we obtained, besides the ganglioside, isolated by Yamakawa, almost the same amount of neuraminic acid-free glycolipid which was closely related to the cerebrosides. But it had a higher content of sugar and contained a disaccharide instead of a monosaccharose, so that it was probably a substance as indicated in Formula I.



I. Glycolipid of the stroma of horse erythrocytes



II. Ganglioside of the stroma of horse erythrocytes

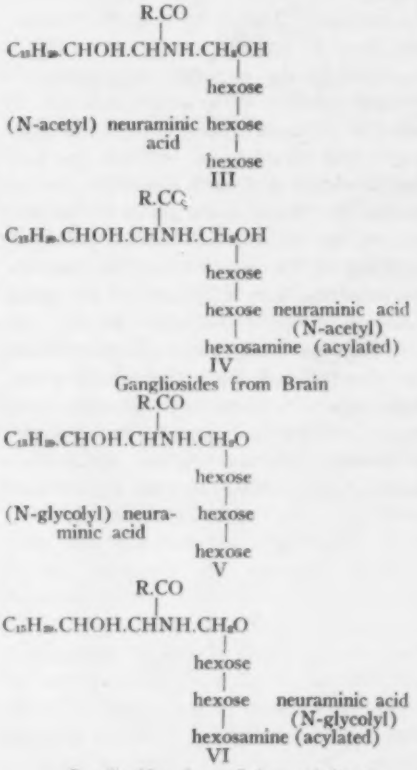
The disaccharide is possibly lactose, for the sugar components among the hydrolytic cleavage products were shown to be both glucose and galactose. The ratio, however, was not exactly 1:1, but somewhat more galactose than glucose has been found. In any case, the properties of this substance are very much similar to those of the cerebrosides and nothing justified the assumption that the substance is present in a polymerized form. It can hardly be doubted that the carbohydrate component is a disaccharide and not a polysaccharide.

The cleavage products of the ganglioside, occurring besides this cerebroside in the horse erythrocytes, correspond to those of the cerebroside in each regard. Both fatty acid and sugar components are the same. An additional component is the acylated neuraminic acid, so that the composition is that shown in Formula II. We have also determined the acetyl content of this ganglioside, since Blix had isolated from mucin a substance, which was similar to neuraminic acid and which was first considered by Blix to be a diacetyl derivative of hexosamine containing disaccharide, but which was later called sialic acid. In our ganglioside, practically no acetyl group was present. From porcine submaxillary mucin, Blix has recently obtained another sialic acid, which was shown to be a glycolyl derivative. We have therefore examined our ganglioside for glycolic acid. The result was positive. We obtained almost one mol glycolic acid per mol of neuraminic acid. After hydrolysing this ganglioside with weak hydrochloric acid we could isolate the well crystallizing glycolyl neuraminic acid, which was in each regard identical with the glycolyl-containing sialic acid obtained from porcine submaxillary mucin by Blix. Afterwards we found out that Yamakawa had

obtained the same result some months earlier.

It seems to me that, on the whole, the structure of this ganglioside is settled, provided that its structure corresponds to that of the cerebrosides. The question, however, whether the neuraminic acid is connected to the first or to the second hexose rest and whether the first or the second hexose rest is glucose or galactose respectively, is still open to further investigations. In any case it seems likely that the carbohydrate group of this ganglioside is at least closely related to the neuramin lactose, a trisaccharide found by Trucco and Caputto in milk and more recently investigated by Kuhn et al. and so we may assume that the first hexose rest is glucose, the second galactose and that neuraminic acid is connected to the galactose. In this trisaccharide of the milk, however, the acetyl neuraminic acid occurs instead of the glycolyl derivative.

If the structure of the gangliosides from horse erythrocytes can be considered to be correct, it is permissible to draw certain conclusions concerning the structure of the brain and spleen gangliosides. They differ from the gangliosides of horse erythrocytes above all, by their hexosamine content. The occurrence of hexosamine in brain gangliosides has first been demonstrated by Blix; however, he thought this hexosamine to be a constituent of an acid disaccharide occurring in the gangliosides as a diacetyl derivative, one of the already mentioned sialic acids, from which our methoxy neuraminic acid should be derived as a decomposition product. We could quickly correct this error. It was apparent that together with the other sugars, the hexosamine was an additional component of the gangliosides besides the neuraminic acid. Since in the gangliosides from brain and spleen the molar ratio of fatty acid, neuraminic acid and hexose (including hexosamine) is 1:1:3, it is reasonable to suppose that they structurally differ from the other gangliosides by a third sugar or a hexosamine rest connected to the second hexose molecule. The structure would consequently be as shown by the Formulas III to VI. There are many indications that the gangliosides both from brain and from spleen are mixtures of hexosamine-containing and hexosamine-free compounds of that kind. First of all the gangliosides from brain and from spleen differ by the nature of the fatty acid components. The fatty acids of the brain gangliosides mainly consist of stearic acid and small amounts of lignoceric and nervonic acid, while the main fatty acids of the spleen gangliosides are lignoceric and nervonic acid, the lower fatty acids occurring only in small amounts. But the two gangliosides are also distinguished by the neuraminic acid, which in the brain gangliosides occurs as an acetyl derivative, while in the spleen gangliosides of beef as well as in the gangliosides of horse erythrocytes the glycolyl derivative is present, as we have found only a few weeks ago.



Elementary Composition of All Gangliosides Isolated

			% C	% H	% N
Erythrocytes of horse					
Cerebrosides (dihexoides)	$C_{54}H_{103}O_{13}N$ (974.37)	calc.	66.56	10.65	1.44
		found	66.53	10.34	1.33 (1.31)
Gangliosides	$C_{61}H_{112}O_{27}N_2$ (1281.63)	calc.	60.91	9.44	2.19
		found	60.37	9.11	2.10 (2.09)
Spleen of beef					
Gangliosides	$C_{72}H_{133}O_{27}N_3$ (1484.8)	calc.	59.05	9.03	2.83
	$C_{71}H_{132}O_{27}N_2$ (1443.8)	calc.	59.06	9.06	1.94
		found	59.3	9.2	2.07
Brain					
Gangliosides	$C_{67}H_{123}O_{28}N_3$ (1384.7)	calc.	58.11	8.81	3.04
	$C_{66}H_{121}O_{28}N_2$ (1343.6)	calc.	58.10	8.85	2.09
Gangliosides (normal)		found	57.1	8.5	2.64 (2.66)
Gangliosides (Tay-Sachs)		found	58.5	8.9	2.74
			(58.5)	(8.8)	

The carbohydrate group of the gangliosides is in some regard similar to the mucopolysaccharides, at least as far as the nature of the components is concerned. In this country one is especially inclined to attribute to the gangliosides a macromolecular structure. It is justified indeed to discuss this question. The view, that the gangliosides have a macromolecular structure, is supported by the fact that they produce a colloidal solution with water and that by means of ultracentrifugation their molecular weight was found to be between one hundred thousand and half a million. In my opinion the strong aggregation of the particles in an aqueous solution is sufficiently explained by the occurrence of the lipophilic groups of the fatty acids and of the sphingosine in the lipid molecule. In any case these findings do not prove the macromolecular structure of the carbohydrate group. With regard to their crystallization from organic solvents, the gangliosides resemble the cerebrosides and similar lipids to a great extent, forming also the typical birefringent spherocrystals. Nothing seems to indicate that the molecular weights of the substances differ essentially, i. e., hundred- or thousand-fold. Some years ago I have assumed—for reasons that need not be discussed here—that two of the basic components might be combined to a double molecule. But for the reasons which I have just mentioned a still higher molecular weight will hardly come into question.

Finally I should like to show you a Table, giving the elementary composition of all the gangliosides isolated by us. All determinations have been carried out at a time when quite a number of the constituents were still unknown, some of them almost 20 years ago. I have also entered the expected values, calculated for the structures shown.*

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* The Bibliography for this article was received too late for insertion at this point. It may be found on page 760.

Serum Phosphatase in Lipidoses

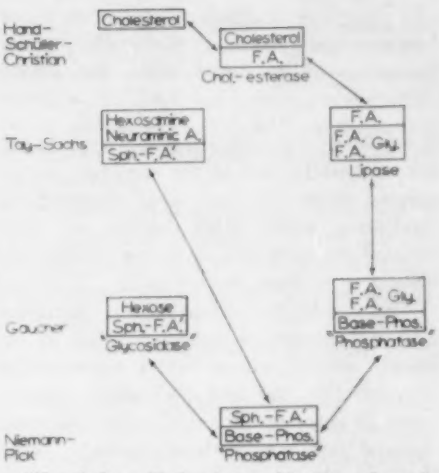
H. SOBOTKA, Ph.D.; G. GOLDSTEIN, Ph.D., and S. WEISSBARTH, Ph.D.

Long before the scientific age in which we live, mankind has striven to evade or to neutralize the mysterious dangers, lurking on all sides, by giving them names through which they may be recognized and identified. This propensity has been rationalized in modern times and one has consciously and proudly pointed to the contributions made by systematic terminology and, more generally speaking, by semantics to science. We have gone from the superstitious adage "nomen est omen" to the Hippocratic maxim "et causae quoque estimatio saepe morbum solvit," and have then often christened a disease after the rationale of its origin.

In the instance of amaurotic family idiocy, the subject of the present symposium, the name is vaguely descriptive, and, if we recognize it as one of the lipidoses or lipid storage diseases, we have gone one step further. Moreover, it is an "inborn error of metabolism," a description which presumes a certain type of mechanism.

If we consider the lipidoses, it will be useful to glance at the composition of the compounds whose storage has given the name to this group of diseases. When we previously discussed the methodological aspects of the analysis of organ lipids,[1] it was pointed out that each of the simpler lipoid building blocks occurs in at least two complex lipids (Figure). Thus, the quantitative analysis of these building blocks still permits an infinity of combinations and does not teach us more than amino acid analysis can tell us about protein structure. Refinements in the procedure of isolation have led to the recognition of the existence of individual complex lipids assignable to given clinical syndromes.

Department of Chemistry, Mount Sinai Hospital, New York.



Abbreviations: F. A.—fatty acid; Gly.—glycerol; F. A.'—lignoceric acid, etc.; Chol.—cholesterol; Base-Phos.—choline phosphate, cholamine phosphate.

The interconversion of lipids can be interpreted in its overall effect as "transesterification" brought about by the hydrolytic and synthetic activity of enzymes. In the lipidoses, the enzymatic regulation of these equilibria is disturbed. Substances, useful under normal conditions as intermediate and transport forms, are not further metabolized, but accumulate in certain organs, especially in the reticulo-endothelial system. When these concepts were first formulated,[2] we thought that all these enzymopathies were in the nature of enzyme deficiencies. This idea was confirmed by studies on the hepatic esterase in various cases of Niemann-Pick's, Gaucher's, and Schueller-Christian's disease at a time when the differentiation of specific esterases was not as far advanced as it is today.[3] The hydrolytic effect of these enzymes upon methyl butyrate is exemplified in Table 1; the low esterase titer in these cases indicated a general

TABLE 1.—*Esterase Values in Glycerol Extract of Chopped Fresh Liver on Methyl Butyrate* *

Normal Liver	1.76	1.60	1.42	
Gaucher's Disease	0.69	0.27	0.23	0.80
Schueller-Christian's Disease	0.27			
Niemann-Pick's Disease	0			
Fatty Degeneration of Liver	0.57			
Liver Atrophy	0.55			
Tuberculous Liver	0.13			

* (cf. ref. 3)

TABLE 2.—*Serum "Acid-pH" Phosphatase in 24 Cases of Gaucher's Disease*
Expressed in King-Armstrong Units/100 ml.
Normal Range 0-4 Units

4.8	5.4	5.5	5.6	5.6	6.4
7.0	7.0	7.1	8.1	8.2	8.3
8.8	8.8	9.3	9.3	9.5	9.6
9.7	9.8	10.2	10.8	11.7	14.3

disturbance of lipid metabolism, but it could not completely explain the accumulation of specific lipids. It was also observed in conditions where other sectors of lipid metabolism were deranged, not always resulting in storage phenomena.

The accidental finding of an increased acid pH serum phosphatase value in an elderly woman, whose history subsequently revealed that she had undergone splenectomy 18 years earlier for Gaucher's disease, induced us to resume these studies.[4,5] Table 2 shows 24 cases of Gaucher's disease with acid pH serum phosphatase values ranging from 4.8-14.3 King-Armstrong Units. The normal range of this determination is from 0-4 Units. Elevated values of this enzyme had hitherto been considered pathognomonic for metastatizing prostatic malignancies.

The increased acid phosphatase values in lipidoses may be distinguished, however, from those in prostatic disease by the performance of an acid phosphatase determination with glycero-phosphate as substrate. The resulting Bodansky units in Gaucher's disease range from 0.2-1.3 units within the normal range, while the Bodansky units in prostatic disease are increased in proportion to the King-Armstrong Units. Since many laboratories determine phosphatase with glycero-phosphate as substrate, they were bound to miss the high phosphatase values in Gaucher's disease.

The effect of the enzyme upon sphingomyelin and other phosphatides remains to be investigated in vitro. Circumstantial evidence strongly suggests that the abnormal amounts of this phosphatase in Gaucher's disease split sphingomyelin beyond the rate prevailing in normal metabolism and that the lignoceryl sphingosin and related acyl sphingosins subsequently enter glycosidic combinations to form excessive amounts of cerebrosides.

We have not yet had the opportunity to study any case of Tay-Sachs' disease. If the serum of these patients follows the same pattern—and we expect it does—this would fall in line with our scheme; in this instance the liberated acyl sphingosin would preferentially combine with hexosamine and neuraminic acid to form gangliosides.

During the early stages of this investigation we became acquainted with a case of Niemann-Pick's disease, published by Hastrup and Videbaek,[6] in which the acid pH phosphatase was likewise elevated. In this instance we assume that the enzyme catalyses the transfer of phosphocholine from lecithin to sphingomyelin at an excessive rate.

The study of the specificity of phosphatases acting upon phospholipids in vitro is incumbered by the lack of solubility of these substrates. We have learned that the acid pH phosphatase in Gaucher's and Niemann-Pick's disease hydrolyses phenyl phosphate, not a very physiological model, but that the same sera show no elevation of phosphatase titer against glycerophosphate. As the Bodansky units (glycerophosphate) are based on the measurement of liberated phosphoric acid and the King-Armstrong units (phenyl phosphate) on that of liberated phenol, it was suggested that one enzyme actually splits both substrates and that a phosphotransferase effect be responsible for the apparent discrepancies between the two sets of figures. Axelrod[7] has demonstrated the presence of an acid pH phos-

phatase in citrus fruit and showed that this enzyme was capable of transferring phosphate from p-nitrophenyl phosphate to a suitable acceptor, e. g., methanol; thus, up to 60% of the hydrolyzed phosphate fails to appear as inorganic phosphate. We determined the liberated phosphoric acid along with the liberated phenol in the hydrolysates of phenyl phosphate in normal and in Gaucher's disease serum. The citrus enzyme furnished, in our hands, an average molar ratio of phenol to phosphoric acid, of 1.30. With Gaucher's and with normal sera this ratio was 0.90 and 0.96; in other words at least as much phosphate as phenol was set free by the serum enzymes.

The more plausible alternative explanation remains, namely, that we are dealing with entirely diverse acid pH phosphatases. This viewpoint is supported by the fundamental differences, found in the effect of various inhibitors upon acid pH serum phosphatases in lipidosis, in prostatic malignancy and in the phosphatase of red blood corpuscles. Table 3 gives the per cent inhibition of phenylphosphate hydrolysis by L-tartrate, formaldehyde and cupric ion.[8] Tartrate inhibits prostatic phosphatase very specifically and completely; formaldehyde and Cu^{++} inhibit red blood corpuscle phosphatase, which in turn remains unaffected by tartrate. The phosphatase in the serum of Gaucher's disease patients is in general much sturdier and shows only minor inhibition with any of these three agents (Table 3).

In discussing our results, I should like to refer to the Symposium on Cerebral Lipidoses which took place in Antwerp in the summer of 1955.[9] A number of European pathologists, histochemists and chemists discussed this group of diseases. When one reads the papers and discussions, one finds that the pathologists are struck by microscopic observations, which differ fundamentally amongst several clinical types, but also within a given clinical picture. They, thus, postulate a great many disease entities, based on unique histological patterns. The interpretations of the underlying chemical events are often based on histochemical methods; Edgar,[10] however, quotes a number of authors (e. g., Glick [11]) on the severe limitations of histochemical analysis of lipid mixtures.

Regardless of discrepancies in method, it is now generally recognized that storage diseases (and also demyelinating diseases) are "enzymopathies" as we had proposed 25 years ago. However, one looks in vain for any enzymatic studies in the Antwerp symposium.

One must distinguish between almost ubiquitous enzymes, such as those studied by Wroblewski which serve as indicators of tissue breakdown ("chemical biopsy"), and, on the other hand, those tissue-specific enzymes which govern the metabolism of a particular tissue system. Returning to the Figure one finds below the various lipids, the enzymes which hydrolyze them. Under physiological homeostatic conditions, synthesis and catabolism will be in equilibrium. If the enzymatic hydrolysis of a lipid along the lines indicated prevails over its synthesis, three things may happen to the split products: (a) they may be sufficiently soluble in the ambient to be carried away and to continue their function elsewhere in the body; (b) they may remain and accumulate, or (c) they may combine with other fragments in the place where they were formed; (d) finally, there may be an excess of synthesis over hydrolysis.

In the first alternative, we shall observe a disappearance or rarification of some tissue elements such as observed in demyelinating disease. In the three other cases, we shall observe storage phenomena; in case (b) simple lipids accumulate as cholesterol in Schueller-Christian's disease. In the

TABLE 3.—*Percent Inhibition of "Acid-pH" Phosphatase Action on Phenyl Phosphate*

	No Inhibitor	L-Tartrate	Formaldehyde	Cu^{++}
Gaucher's Disease	0	15	36	20
Serum Prostata	0	86	6	8
Serum RBC's	0	0	98	53

other cases some type of lipids may be stored, often at the expense of related lipids. Cholesterol, fatty acids, glycerides, phosphoric acid, choline, and carbohydrate are members of other metabolic chains or cycles, but the biogenesis and catabolism of sphingosin, in keeping with its name, is still a riddle.[12]

The phosphatase and the hitherto undiscovered glycosidases, which govern the metabolism of sphingolipids, are subject to genetic disturbances. The abnormally decreased hydrolysis of a particular lipid will lead to its storage, but the excessive hydrolysis of a lipid will lead to accumulation of its chemical "neighbors" on either side in the scheme. Thus, such enzymopathies will rarely affect a single lipid, but will upset the entire lipid equilibrium, leading to more or less typical situations. That some of these are less typical is witnessed by the numerous borderline cases, like juvenile Gaucher's disease or a case described as Niemann-Pick's disease in a 40-year-old man[13] and the frequent overlapping of Niemann-Pick's with Tay-Sachs' disease.

A future taxonomy of these diseases, based on their enzymatic etiology, will distinguish between conditions with excess and with deficiency of specific enzymes. One will also have to consider whether the lipid complexes are neurogenic or imported into the CNS from other organs. It will teach us that the symptomatic overlapping of two diseases, especially of their pathological "phenotypes," does not necessarily mean the fortuitous simultaneity of two disease entities, but rather the simultaneity of diverse symptoms of a given single enzymatic dysequilibrium. The refinements of the pathologist's findings will not be lost, but they will not so much determine the classification, but the prognosis. For, the clinical picture depends on which organs, tissues, and cells are hit and not so much on what hit them. The time seems ripe for an approach that emphasizes the common or related causes rather than the divergencies of morphological details. The genetic overlapping of idiopathic hyperlipemia and hypercholesteremia suggests a common enzymatic cause which may give rise to at least two morphologically and clinically diverging manifestations. In general the morphologist will tend to split, while the biochemist and enzymologist are more likely to combine disease entities.

One more word about the possible practical consequences. I am not too optimistic about therapeutic measures for the cure or alleviation of these irreversible changes. But a more advanced knowledge of the enzymes that cause these changes, may in the future permit us to recognize the carriers of the respective genes and help to dissuade them from intermarriage and propagation.

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Plasma Lipoproteins in the Lipoidoses

Changes After a Special Dietary Regimen

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Introduction

A number of investigators, over the years, have demonstrated that in amaurotic family idiocy (Tay-Sachs' disease) some abnormality in lipid metabolism is likely.[1] If this disease may be classified as another resulting from an "inborn error of metabolism," the indications at present are that the error is intimately related to the lipids; whether through genetic channels (suggested by the name and incidence in families), through embryologic anomalies, or through post-natal factors, is not definitely known.

Recent advances in our knowledge of lipid metabolism promise to further the solution to this problem just as, for example, the newer developments of amino acid metabolism have elucidated the abnormality in phenylketonuria.[2] It is now quite generally accepted that lipids, whether dietary or metabolic, are transported, at least in man, as lipid-protein or lipid-carbohydrate complexes or both. The various lipids are associated with albumin and the globulins, characterized electrophoretically as alpha-globulin and beta-globulin.[3] As they proceed through the post-absorption state the predominantly lipid molecules which are quite low in density for their size and molecular weight, are believed to become less lipid and more protein with an increase in density and decrease in molecular weight. The question of whether the beta-lipoproteins are metabolized directly to alpha-lipoproteins is not settled.

It is now believed that lipoprotein lipase functions in these changes in blood lipids, serving to "clear" the plasma of optically visible lipids (chylomicrons). Korn has shown that both alpha-lipoprotein and beta-lipoprotein, but especially alpha-lipoprotein, are essential for the activation of the lipoprotein lipase.[4]

With the evidence at hand for the well-ordered scheme of lipid metabolism, probably made possible by lipoprotein lipase, or unelucidated enzymes or both, it is obvious that any break in this train of events might cause as much disaster as is observed in other metabolic abnormalities. Using the available techniques for studying plasma lipoproteins, we are able to present data on three children with different types of lipoidoses, Tay-Sachs', Niemann-Pick's and Gaucher's diseases, and on the parents of the child with Tay-Sachs' disease. A special diet was devised[5] and the changes in the blood lipoproteins of the children are reported.

Procedures for Studying Plasma Lipoproteins

In order to determine whether, in the Tay-Sachs patient and other lipoidoses, lipoprotein metabolism is abnormal, it was necessary to focus on some of the individual metabolites or groups of them as sharply as possible. Fortunately in the past few years excellent methods have become available for the more detailed study of blood components. With particular respect to lipopro-

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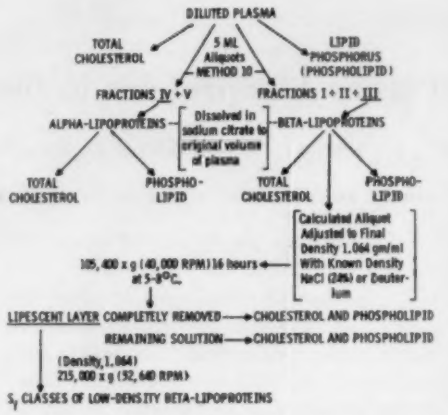
teins, Lever and his co-workers [6] adapted Method 10 of Cohn and his collaborators [7] to small samples of plasma. This provided a means of separating the various kinds of proteins in their more native states. They may then be characterized still further and their exact quantities more nearly determined. This is especially advantageous in studying the proteins which are associated with the lipids and tend to solubilize them in the aqueous blood fluid. Once separated, the individual lipid components of the alpha-globulins and beta-globulins may be conveniently estimated chemically.

A further refinement of the study of lipids as lipoproteins was stimulated by the observation of Pedersen in 1945-7 [8] that the addition of saturated salt solutions to plasma or serum before centrifugation at high speeds caused some component(s), termed by Pedersen "X-protein," to float, like cream, against a gravitational force. Gofman et al. refined this technique and opened the way for greatly detailed studies of the many lipoproteins existing in blood under different circumstances.[9]

A combination of these procedures served as a very useful tool for our work with children from whom only small samples of blood could be taken conveniently. If 12.5 ml. of *fasting* blood (femoral puncture) are added to 2 ml. of acid-citrate-dextrose [6] solution in a cold cylinder, about 7 ml. of diluted plasma may be obtained. This will provide one 5-ml. aliquot for Method 10 and samples for total nitrogen. If possible, it is advisable to increase these volumes and analyze duplicate samples. About 5 ml. of blood added to 1 mg. heparin powder will provide undiluted plasma for determination of total nitrogen. A dilution factor is obtained from a ratio of the two nitrogen values. The Figure shows an outline of the procedures used in this study.


DILUTED PLASMA
TOTAL CHOLESTEROL
5 ML Aliquots METHOD 10
LIPID PHOSPHORUS (PHOSPHOLIPID)
FRACTIONS IV + V
FRACTIONS I + II + III
ALPHA-LIPOPROTEINS
Dissolved in sodium citrate to original volume of plasma
BETA-LIPOPROTEINS
TOTAL CHOLESTEROL
PHOSPHO-LIPID
TOTAL CHOLESTEROL
PHOSPHO-LIPID
Calculated Aliquot Adjusted to Final Density 1.064 gm/ml With Known Density NaCl (24%) or Deuterium
105,400 x g (40,000 RPM) 16 hours at 5-8°C.
LIPESCENT LAYER COMPLETELY REMOVED → CHOLESTEROL AND PHOSPHOLIPID
REMAINING SOLUTION → CHOLESTEROL AND PHOSPHOLIPID
(Density, 1.064)
215,000 x g (52,640 RPM)
S_f CLASSES OF LOW-DENSITY BETA-LIPOPROTEINS

Subjects Studied

Tay-Sachs' Disease.—The female patient's birth weight was 9 pounds, 12 ounces and she was thought to have a mild calcium deficiency neonatally. Calcium was administered orally up to the third week of life.

At the age of 3 months, although she appeared to be developing normally, an ophthalmologist, consulted because of the family history, noted the presence of bilateral cherry-red spots in both macular areas.

She was admitted to the Pediatric Service at the age of 6 months for observation. Examination showed a plump, contented though apathetic baby weighing 19 pounds. The temperature, pulse, and respiration were normal. She was unable to sit or pull herself up and grasped weakly at objects. The reflexes were hyperactive. The cherry-red spots were observed in both fundi. Although she had some vision, it was considered to be impaired.

At 5 months of age her plasma lipoproteins were studied, and she was subsequently placed on the diet illustrated in Table 3. Her plasma lipoproteins were again studied after 4 months on the diet.

From the date she was first observed her course was one of slow general decline. She had reduced motor activity and diminishing attempts to focus her eyes. She ate satisfactorily, except for the liver, which was not well accepted.

At 9.5 months her lipoproteins were again studied. Her weight at 1 year of age was 22 pounds, 10 ounces. At 15 months of age she developed acute tracheobronchitis and was hospitalized again. Recovery from this was uneventful but she became progressively weaker and expired at the age of 21 months.

The family history is contributory. The parents are descendants of Jewish parentage from widely separated areas of Poland and Russia; they are not related. Their first child, a male about 10 years old, is clinically free of disease, but had coeliac disease as an infant. The second male child had Tay-Sachs' disease and died of pneumonia at 22 months.

Niemann-Pick's Disease.—The female patient was the product of a normal pregnancy and

weighed 7 pounds at birth. When first examined at 2.5 weeks, she weighed 7 pounds, 8 ounces. The head circumference was 14 inches, chest 13, abdomen 14, and length 21 inches. She was active, had a good cry, and aside from transient lateral oscillations of the eyes, presented no unusual findings. These ocular signs continued throughout her period of observation. Enlargement of the liver was noticed first at 8 weeks; the edge was 3 cm. below the costal margin, and gradually descended during her first year to 5 cm. below the costal margin.

She was seen in consultation by an ophthalmologist whose examination of her fundi at 3 months did not reveal any cherry-red spots.

The knee and ankle jerks, which had been active, diminished after the third month and were absent after the fifth month.

Her blood lipoproteins were studied at 4 months. At 4.5 months she was placed on a diet free of animal fats, illustrated in Table 3.

She could pull herself into a sitting position at 5 months, and her weight and height at 6.5 months were 17 pounds and 26.5 inches, respectively.

Enlargement of the spleen first occurred at 7 months of age and this gradually increased until the edge was felt 3.0 cm. below the costal margin at 1 year of age. Her first teeth erupted at 7 months, and she talked slightly at 10 months. No cherry-red spots were visible at 9 months.

After 5 months on the diet, the plasma lipoproteins were studied again. She gradually developed increasing muscular weakness after the 8th month. At 16 months of age she weighed 20 pounds, 12 ounces, and her height was 29 inches. She could not raise her head, her cry was fretful, but she appeared able to see. There was no adenopathy. The liver was palpable 6.0 cm. and the spleen 4.0 cm. below the costal margin.

The family history of the patient is contributory in that a female infant sibling had succumbed in 1948 to Niemann-Pick's disease. A male child, born six years before our patient, has been clinically normal. The parents are both Jewish and are not related.

Gaucher's Disease.—At the time this male patient was first seen by one of us (H. W. D.), he was over 5 years of age and known to have Gaucher's disease. He first came under medical care for this disease at age 3 when the abdomen was observed to be enlarging and he suffered from epistaxis. Marrow examination had revealed Gaucher's cells, and a splenectomy was performed. He was fairly well for a year when he had severe pains in the right arm and, one month later, the right leg was first affected. Eight months later there was fracture of the right femur.

At age 5.3 years he weighed 47 pounds and had to be carried. He held his hips in semi-flexion (45°) and there was limitation of motion in the left shoulder girdle. X-ray showed typical signs of Gaucher's disease in both femurs with evidence of fractures in the left and right femoral necks. The surgical neck of the left humerus also showed disease and fracture.

Blood lipoprotein studies were carried out at this time and the patient placed on the special diet (Table 3). He was also given 50 mg. of 6-mercaptopurine per day, and several months later 3 to 4 capsules of Methiscol * were added to the dietary regimen. The 6-mercaptopurine administration was stopped 10 days before the second lipoprotein study was done, after 4 months on the diet.

He has suffered severe crises at 2- to 3-month intervals since.

The family history of the patient is contributory. One paternal uncle died at 4 years of age from Gaucher's disease and another has the disease but is still living at 28 years of age.

Control Subjects.—Two babies, aged 7 months, were available, and the data were averaged.

The adult women in the same age group as the mother had been subjects of another study in our laboratory.[10] The data on adult males are from the literature.[11]

The blood samples from all the subjects were taken in the morning and analyzed immediately. The patient with Tay-Sachs' disease had been given a light breakfast of apple sauce before being brought in to the hospital. None of the other subjects had had breakfast.

Experimental Results

Quantities of Lipoproteins in Plasma from the Patient with Tay-Sachs' Disease.

The two types of lipids, total cholesterol and phospholipid, studied chemically in the plasma, and the quantities of these associated with the alpha-lipoprotein and beta-lipoprotein fractions, are shown in Table 1. Data from control babies are given for comparison. If the "before diet" data are considered first, it may be observed that this patient did not have abnormally great quantities of total cholesterol or phospholipid in the whole plasma according to our control values.

* Methiscol (U. S. Vitamin Corporation). Constituents per capsule: choline dihydrogen citrate 0.83 gm.; *DL*-methionine 0.33 gm.; inositol, 0.25 gm.; liver concentrate and desiccated liver 0.26 gm.; vitamins B_{12} and B_{mb} 6.00μg.

TABLE 1.—*Tay-Sachs Disease: Lipids in Plasma; Alpha- and Beta-Lipoproteins Before and After a Therapeutic Diet*

Subjects and Components	Plasma Mg. %	Alpha-Lipoprotein Mg. %	Alpha-Lipoprotein % of Total	Beta-Lipoprotein Mg. %	Beta-Lipoprotein % of Total
Tay-Sachs					
Cholesterol					
Before	248	42	17	197	79
After	132	35	23	111	73
Phospholipid					
Before	251	86	34	154	61
After	196	76	39	98	50
Control					
Cholesterol	223	41	18	182	83
Phospholipid	215	98	46	113	52

Chemical analyses on the alpha-lipoprotein fractions showed that the absolute quantity, as well as the proportional quantity of cholesterol in this fraction was not different from the control values. In the case of phospholipid, however, lower quantities were present in the patient, although it should be noted that a greater amount was present in the whole plasma of the patient.

More pronounced differences from the normal appear in the beta-lipoprotein fraction which is more abundant, as evaluated by both cholesterol and phospholipid determination in the patient.

An interesting point in the results is the differences observed in the distribution of the total plasma phospholipid between the alpha-lipoprotein and beta-lipoprotein fractions. In a number of normal adult subjects studied by these methods,[10] the phospholipid is consistently distributed about 50 per cent in each fraction. This is observed also in the control babies (46 per cent in alpha-lipoprotein and 52 per cent in beta-lipoprotein), but not in the patient with Tay-Sachs' disease (34 per cent and 61 per cent, respectively), in whom the beta-lipoprotein fraction is large.

The quantity of beta-lipoprotein in the patient, estimated by chemical means, is corroborated by the large amounts of beta-lipoprotein of density less than 1.064 gm/ml. determined both by chemical and physical procedures. These data are presented in Table 2. The lipescent top layer, removed after 16 hours of ultracentrifugation, contained 176 mg. per cent cholesterol compared with 144 mg. per cent in the control subjects. The low-density beta-lipoproteins are shown in four S_f classes; it will be seen that there were notably greater quantities of all the S_f classes in the plasma from the patient. Of especial importance is the presence, though small, of the S_f class 101-400 which is not usually observed in the blood of fasting normal individuals. In addition, the amounts of S_f classes 11-20 and 21-100 are quite high compared with control subjects.

The figures for cholesterol in the first and last columns of Table 2 serve to check the chemical with the physical data. Since approximately 33 per cent [12] of the total quantity of low-density beta-lipoprotein has been shown to be cholesterol, the agreement in these figures adds confidence to data obtained, of necessity sometimes, on small numbers of cases.

These lipoprotein data, obtained on a patient with clinically proven Tay-Sachs' disease, indicated to us that a metabolic defect in lipid metabolism was quite probable; and that such measurements as these, especially the determination of the S_f classes of low-density lipoproteins, would help to

TABLE 2.—*Tay-Sachs Disease: Beta-Lipoproteins: Physical and Chemical Measurements Before and After a Therapeutic Diet*

Subjects	Low-density Beta choles. Mg. %	S_f Classes Mg. % 0-10	11-20	21-100	101-400	Total	Choles. in Total S_f Mg. %
Tay-Sachs							
Before	176	452	32	61	6	552	182
After	98	332	25	24	0	281	93
Control	144	414	13	10	0	437	144

TABLE 3.—*Dietary Regimen*

	Total Grams	Composition in Grams: Protein	Fat	Carbohydrate	Total Calories
Soybean Milk—"Mull-Soy" (Mixed)	930.0	27.8	37.2	41.8	620
Cereal—"Pablum"	74.0	10.1	2.2	50.0	262
Liver	5.0	1.0	0.5	0.2	10
Banana	50.0	0.5	0.1	11.0	65
Cotton Seed Oil	10.0	0.0	10.0	0.0	98
Prunes	120.0	0.9	0.2	28.9	119
Essential B Vitamins—"Vi-Penta"	0.3	0.0	0.0	0.0	0
Cevex	0.6	0.0	0.0	0.0	0
Orange Juice	90.0	0.0	0.0	10.0	41
Total Composition in Gms.		40.3	50.2	141.9	
Calories from Protein, Fat and Carbohydrate		161.1	466.9	581.8	
Total Calories					1,210
Per Cent Calories from Protein, Fat and Carbohydrate		13.0	39.0	48.0	

find the metabolic error, if such exists, in these patients.

A possible two-edged sword in other metabolic disorders has been controlled food intake.[13,14] Therefore, we devised the dietary regimen presented in Table 3. This diet was designed to provide adequate quantities of protein, carbohydrate, vitamins, minerals, bulk, calories,[5] and a good source of the essential fatty acids [15] for a growing child. The fat was to be provided from vegetable sources [16,17] and a good source of choline and methionine was provided.[18] The liver first used was later substituted by "Methiscol," * 2 capsules daily, which was more easily administered and more readily accepted, since acceptability of any therapeutic diet is a major problem.

After the child had been on this diet for four and one-half months, another fasting blood sample was studied. The results are shown in Tables 1 and 2. The absolute quantities of every component studied were lowered. Since the values for the low-density beta-lipoproteins were the most abnormally high in the pre-dietary study, the decreased quantities of these after the diet were the most noteworthy. The S_f class 101-400 lipoproteins were eliminated from the blood and more normal values for S_f classes 21-100 and 11-20 were observed. The agreement between the quantities of cholesterol by chemical analyses and as 33 per cent of the total S_f classes was improved.

Clinical course of the patient with Tay-Sachs' disease. Unfortunately, the patient did not undergo clinical improvement so far as the manifestations of her disease were concerned; however, she continued to eat well and gain weight so that the lower quantities of lipoproteins are not the result of extensive weight loss. She survived just under two years. It is impossible to state whether she would have declined more rapidly had no dietary restriction been imposed.

Lipoprotein Studies on the Parents of the Tay-Sachs Patient

It is now established that in certain metabolic disorders, abnormalities may be detected in the parents in which no evident symptoms prevail. Improved techniques have enabled interested investigators to detect quantities of "abnormal" metabolites so low as to cause no clinical symptoms but nevertheless to indicate that such manifestations may be potential or latent.[2]

We thought it advisable to study the plasma lipoproteins in the parents of the patient with Tay-Sachs' disease because of the many previous indications that a hereditary relationship may exist and the additional fact that, in this family, a sibling had died of this disease.

TABLE 4.—*Parents of Tay-Sachs Patient Lipids in Plasma; Alpha- and Beta-Lipoproteins*

Subjects and Components	Plasma Mg. %	Alpha-Lipoprotein Mg. %	Alpha-Lipoprotein % of Total	Beta-Lipoprotein Mg. %	Beta-Lipoprotein % of Total
Cholesterol					
Mother	257	57	22	206	80
Controls	208	55	27	145	70
Father	233	51	22	174	75
Controls	197	50	25	142	72
Phospholipid					
Mother	251	100	40	131	52
Controls	241	122	50	117	49
Father	226	103	46	124	55
Controls	195	83	42	87	45

TABLE 5.—*Parents of Tay-Sachs Patient Beta-Lipoproteins: Physical and Chemical Measurements*

Subjects	Low-density Beta choles. Mg. %	S_f Classes Mg. %					Choles. in Total S_f Mg. %
		0-10	11-20	21-100	101-400	Total	
Mother	154	396	34	60	0	490	162
Controls	114	260	26	29	0	315	104
Father	137	338	42	46	11	427	141

Tables 4 and 5 contain the results from the parents and from control subjects.

Although both the parents had greater amounts of cholesterol and phospholipid in the plasma and beta-lipoprotein fraction than their respective controls, the values cannot be considered out of the normal range. The low-density beta-lipoprotein values (Table 5) indicate a possible clue to unusual lipid metabolism in the mother who has greater quantities of the S_f classes than the control subjects. A value of 60 mg. per cent of the S_f class 21-100 is quite high for a young woman with active estrogen function.

Possible Relationship of Tay-Sachs' Disease with Niemann-Pick's and Gaucher's Diseases

These disorders have been considered together as the lipoidoses [1] for the reason that lipid abnormalities have been demonstrated in all of them. These abnormalities vary and details are beyond the scope of the present paper. We have had the opportunity to study the lipoproteins in the three types of lipoidoses cited. Three possible comparisons follow: the relationship to control subjects; to each other, and the responses of the three diseases to the diet. All are presented in Tables 6, 7, and 8.

In Table 6 the cholesterol results are presented. The values obtained before the diet are, in general, quite different. Whereas both the Tay-Sachs and Niemann-Pick patients had greater amounts of plasma cholesterol than the control subjects, the Gaucher's patient had a lower amount. This was also true with beta-lipoprotein cholesterol. A difference is noted in alpha-lipoprotein cholesterol values: the Niemann-Pick and Gaucher's patients had quite the same quantities of alpha-lipoprotein but only one-half that of the Tay-Sachs patient. However, the proportional quantities differed, especially in Niemann-Pick's disease.

The response of the three patients to the diet was one of a general lowering of cholesterol in plasma and its fractions, but in Niemann-Pick's and Gaucher's disease there appears to be a transfer of cholesterol from the alpha-lipoprotein to the beta-lipoprotein.

The phospholipid values in Table 7 are possibly more interesting. Again, the pre-

TABLE 6.—*Cholesterol: In Plasma; Alpha- and Beta-Lipoproteins in Three Lipoidoses Before and After a Therapeutic Diet*

Subjects	Plasma Mg. %	Alpha-Lipoprotein Mg. %	Alpha-Lipoprotein % of Total	Beta-Lipoprotein Mg. %	Beta-Lipoprotein % of Total
Control	223	41	18	183	83
Tay-Sachs	248	42	17	197	79
	152	35	23	111	73
Niemann-Pick	299	18	6	269	90
	271	2	1	244	90
Gaucher's	158	21	13	137	87
	137	3	2	130	95

TABLE 7.—*Phospholipid: In Plasma; Alpha- and Beta-Lipoproteins in Three Lipoidoses Before and After a Therapeutic Diet*

Subjects	Plasma Mg. %	Alpha-Lipoprotein Mg. %	Alpha-Lipoprotein % of Total	Beta-Lipoprotein Mg. %	Beta-Lipoprotein % of Total
Control	215	98	46	113	52
Tay-Sachs	251	86	34	154	61
	196	76	39	99	50
Niemann-Pick	317	69	22	246	78
	405	93	23	300	89
Gaucher's	222	51	23	171	77
	158	37	23	126	79

TABLE 8.—*Beta-Lipoproteins: Physical and Chemical Measurements in Three Lipoidoses Before and After a Therapeutic Diet*

Subjects	Low-density Beta choles. Mg. %	S_f Classes Mg. %					Choles. in Total S_f Mg. %
		0-10	11-20	21-100	101-400	Total	
Control	144	414	13	10	0	437	144
Tay-Sachs	176	452	33	61	6	552	182
	98	232	25	24	0	281	93
Neimann-Pick	230	625	197	292	49	1163	384
	176	651	101	169	0	921	304
Gaucher's	128	302	38	33	0	373	123
	118	271	68	68	0	407	134

dietary values are quite different, especially those observed in the patient with Niemann-Pick's disease in whom higher quantities of both plasma and beta-lipoprotein phospholipid were observed. The distribution of phospholipid between the alpha-lipoprotein and the beta-lipoprotein fractions is identical in the patients with Niemann-Pick's and Gaucher's disease but is not like that in Tay-Sachs' disease. None can be considered a normal distribution such as is seen in the control subjects.

The patient with Tay-Sachs' disease and the patient with Gaucher's disease responded quite similarly to the diet. In contrast to the general pattern the patient with Niemann-Pick's disease had greatly elevated quantities of phospholipid in plasma and the fractions studied. The distribution of phospholipid changed very little in any of the subjects.

The most startling results were obtained when the low-density beta-lipoproteins were measured in these three types of lipoidoses. It can be seen in Table 8 that they differed rather markedly. The most extreme values were observed in the patient with Niemann-Pick's disease; all the S_f classes studied were very greatly elevated. On the other hand, and with the exception of S_f classes 11-20 and 21-100, the patient with Gaucher's disease had even lower quantities of these low-density beta-lipoproteins than the control subjects.

The dietary regimen resulted in lower values for these beta-lipoproteins, with the notable exception of S_f classes 11-20 and 21-100 in the patient with Gaucher's disease.

At least so far as the lipoprotein studies are concerned, it cannot be denied that in these three patients with lipid abnormalities, notable differences were observed in the values before and after dietary regimen was instituted.

Clinical Response of the Three Types of Lipoidoses to the Diet

Although there was no clinical improvement from the diet, in the sense that the symptoms of the disease were allayed, it is interesting to note that these children continued to gain weight and grow in stature. The weight gain is probably the result of deposited lipids rather than an increase in muscle tissue. The neurological symptoms progressed and there was gradual progression in the size of the liver and spleen in the patients with Tay-Sachs' and Niemann-Pick's disease.

Comment

It was hoped that a rather detailed study of some of the different plasma lipids and lipoproteins in these patients would uncover unusual quantities of them if such existed. No attempt has been made to search for "abnormal" components. During the past few years numerous investigators, using blood fractionating techniques and ultracentrifugal methods, have been able to show that in certain diseases there are significant aberrations in the quantities of plasma lipids and lipoproteins from the normal. Out of these studies has derived a rather

well-defined pattern of what is usually found in the person clinically free from disease.

The generally accepted normal pattern in adults has been shown to be dependent upon many physiological processes and metabolites, including hormones, particularly the sex hormones. In general, in young adult women averages of 55-60 mg. per cent alpha-lipoprotein cholesterol and 145-150 mg. per cent beta-lipoprotein cholesterol (25 per cent of the total cholesterol in the alpha-lipoprotein fraction and 75 per cent in the beta-lipoprotein fraction) are considered desirable. Phospholipid is distributed rather equally, and unfortunately, has not been studied in these lipoprotein fractions so widely as cholesterol has. In young men somewhat less cholesterol is found in the alpha-lipoprotein fraction and more in the beta-lipoprotein. High values for S_f 11-400 are considered undesirable in adults and certainly in children.[19]

So far as the plasma lipoprotein values in these three examples of the lipoidoses are concerned, the alpha-lipoprotein and beta-lipoprotein concentrations are abnormal when compared with values from our control subjects and even with normal adults. However, the degrees of abnormality are quite different. It is doubtful that sex alone would cause these differences. Even though only one case of each type of the lipoidoses was studied, some of the differences are too great to be sampling errors only. This is especially true in the patient with Niemann-Pick's disease who had very greatly elevated quantities of the low-density beta-lipoproteins, and almost no alpha-lipoproteins. These results are in complete agreement with those of Pansky and Lee[20] who, with different techniques, observed essentially the same values, even though their patient was a few months older.

It has been stated that the lipid "accumulated in each disease is characteristic for that disease, but is often accompanied by slight disturbances in the metabolism of other lipids."[1] In these diseases abnormalities in phospholipid metabolism have long been suspected and rather recently demonstrated, so it is not surprising to find the very low quantities of phospholipid in the alpha-lipoprotein fraction. Since the alpha-lipiproteins "contain" a greater proportion of the plasma phospholipid than the beta-lipoproteins (which "contain" a greater proportion of the total cholesterol), two possible interpretations arise. Either these patients have low circulating phospholipid or it is associated, in these cases in contrast to normal individuals, with the beta-lipoprotein fraction, in which very great quantities were observed. The latter is likely to be the more correct view because in all three patients disproportionately high percentages of the total plasma phospholipid were associated with the beta-lipoprotein fraction. The patients with Niemann-Pick and Gaucher's diseases were outstanding in this respect, and in both of these phospholipid metabolism is believed to be abnormal. There may be some relationship between the very low alpha-lipoprotein phospholipid in the plasma of the patient with Gaucher's disease and the fact that his disease was definitely of the osseous type with accompanying fractures. Similar low alpha-lipoprotein phospholipid values are seen in patients with advanced mammary carcinoma and osseous metastases.[21]

Although the patients with Tay-Sachs' and Gaucher's diseases had elevated beta-lipoproteins, their values for the S_f classes of lipoproteins cannot compare with the extremely high values observed in the patient with Niemann-Pick's disease.

Certainly when these components are studied and compared, a difference is apparent in the lipoproteins in the three lipoidoses.

Nor did the patients' blood lipid values respond in the same way to the dietary regimen. Though, on the whole, the lipoproteins were lowered, the phospholipid values in the patient with Niemann-Pick's disease were greatly increased.

As stated in the history resume of the patient with Gaucher's disease, this patient was given 50 mg. daily of 6-mercaptopurine

after the first lipoprotein study was carried out. However, he continued intermittently to receive this drug until 10 days before the post-dietary lipoprotein study was made, four months after the first study. This patient was unique in that after the diet no decrease occurred in beta-lipoprotein cholesterol, and indeed there was a 100 per cent *increase* in the S_f classes 11-20 and 21-100 low-density beta-lipoproteins. Since there is some evidence in tissue culture [22] and in mice bearing Sarcoma 180 [23] that this compound affects lipid metabolism, this observation is quite intriguing in the patient with Gaucher's disease.

The one instance in which the plasma lipids of the parents were studied and found to be abnormal would not be sufficient to prove that a single gene exerts a measurable effect. However, there is some suggestion that in the mother the low-density beta-lipoprotein S_f classes 11-20 and 21-100 are higher than normal.

No claim can be made for the dietary regimen so far as clinical improvement of the patient is concerned. It was hoped that in the patient with Niemann-Pick's disease, the restricted diet at an early age might prove more beneficial than in instances where the children were older. Perhaps a diet containing even less fat of any origin would be advisable although it is doubtful that restricting fat intake will be the main solution to the problem since fats may be synthesized so readily from carbohydrates. In both the Tay-Sachs' and Niemann-Pick's disease patients, there was an increase in height and weight, and the changes in plasma lipoprotein values cannot be thought to arise from inanition. The fact that the concentrations of some of the components studied after the diet more nearly resembled the normal concentrations indicates that perhaps some control of the disease may eventually arise through these means.

The fact that the S_f classes of beta-lipoproteins were elevated in these patients might offer some suggestion that metabolic processes, and enzyme reactions, were abnormal. Certainly a study of such enzymes as lipoprotein lipase in the blood of these patients is indicated.

Summary

The plasma lipoproteins have been studied in three types of lipoidoses: Tay-Sachs', Niemann-Pick's and Gaucher's diseases. The plasma lipoproteins in the parents of the patient with Tay-Sachs' disease have been studied. The patients were placed on a controlled dietary regimen, for several weeks, and the plasma lipoprotein studies repeated.

1. *Alpha-Lipoprotein Cholesterol.* — The value for this component of plasma was the same in both the control subjects and the Tay-Sachs patient before the diet. In patients with Niemann-Pick's and Gaucher's disease it was very low before the diet was begun. After the diet, this component was only slightly decreased in the patient with Tay-Sachs' disease, but was almost absent in the patients with Niemann-Pick's and Gaucher's diseases.

2. *Alpha-Lipoprotein Phospholipid.*— This component was low in all three patients, in this order: Tay-Sachs, Niemann-Pick, and very low in Gaucher's disease. After the diet, it was decreased only slightly in Tay-Sachs' and Gaucher's diseases, but was increased about 70 per cent in Niemann-Pick's disease.

3. *Beta-Lipoprotein Cholesterol.*—Before the diet, this component was elevated in Tay-Sachs', and was very greatly elevated in Niemann-Pick's disease. It was rather below the control value in Gaucher's disease. After the diet, this component was decreased markedly in Tay-Sachs', slightly in Niemann-Pick's, and not at all in Gaucher's disease.

4. *Beta-Lipoprotein Phospholipid.*—This component was higher than the control value in Tay-Sachs' and Gaucher's diseases, and appreciably higher in Niemann-Pick's disease. After the diet, it was reduced in Tay-Sachs' and Gaucher's diseases, but markedly elevated in Niemann-Pick's disease.

5. *Low-Density Beta-Lipoprotein S_f Classes.*—All were elevated markedly in

Tay-Sachs' and extremely so in Niemann-Pick's disease before the diet, and were lower after the diet. In Gaucher's disease only the S_f classes 11-20 and 21-100 were abnormally high before the diet, and doubled in concentration after the diet.

6. It may be concluded that in these three lipoidoses abnormal quantities of the plasma lipoproteins studied have been found. The degrees of abnormality were not the same in the patients before the dietary regimen was imposed, nor was the response to the diet the same in all cases.

7. No outstanding lipoprotein abnormality was observed in the parents of the Tay-Sachs patient, with the possible exception of elevated values for S_f classes 11-20 and 21-100 in the mother.

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Studies on the Brain Ganglioside Strandin in Normal Brain and in Tay-Sachs' Disease

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Since our results bearing directly on Tay-Sachs' disease are few and mainly of a confirmatory nature, it would seem that the best contribution that we can make to this Symposium is to report in some detail our own work on the brain ganglioside, strandin. This will constitute the bulk of the discussion and will be followed by the presentation of the results of the analyses of two brains of patients with Tay-Sachs' disease.

Strandin [1] was reported originally as a complex lipide which had been isolated from gray matter by three different methods. Its name was chosen because, when recovered from water solutions by drying, strandin was obtained as a residue of long, glistening, birefringent strands. It was readily soluble in water as an undialyzable solute which was homogeneous in the electrophoretic field over a wide pH range. By ultracentrifugation it showed a major component which accounted for 80% of the material and which had a particle weight of >250,000. The first preparations obtained were soluble in a number of organic solvents or mixtures of solvents, but these solubilities decreased on purification with the result that the purest preparations available were insoluble or only sparingly soluble in organic solvents. Strandin contained 2.6% N and was a monobasic acid with a neutral equivalent value of ca. 1,500. It was shown to be constituted by at least five constituents, or types of constituents, namely, fatty acid(s), sphingosine or a sphingosine-like substance, neutral sugar(s), a primary amine which gave the reactions of a hexosamine, and another radical, different from the preceeding four, which darkened on treatment with hot aqueous mineral acids and which was named "chromogenic group" not only because of its "browning" reaction but also because of its ability to give colored products with a number of different reagents. The browning reaction was developed into a method for the quantitative estimation of strandin. It also provided a useful tool for the operational identification of strandin in mixtures of unknown composition. Strandin was non-reducing and contained no free primary amino groups. The preparations obtained by the three different methods of isolation were similar in physical properties and chemical composition with the exception that strandin obtained by the "partition dialysis" method contained a small amount of combined amino acids (0.3%-0.6% α-amino acid N after acid hydrolysis) which was absent from strandin prepared by the other two methods. All preparations were contaminated with small amounts of phosphatides (0.2%-0.4% P) and sulfatides (<0.2% S). Through an analytical error, strandin was reported to contain <1.5% neuraminic acid, but it was soon shown by Daun [2] and confirmed by Chatagnon and Chatagnon [3] that neuraminic acid was present in strandin in substantial amounts and that it was most likely the chromogenic group. This error resulted in the belief that strandin was radically different from the gangliosides obtained by Klenk [4] ten years earlier, and the difference appeared to be established further by the presence, in strandin, of a hexosamine, which, at that time, was supposed to be absent from gangliosides.

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After the publication in 1951 [1] of the observations summarized above, the study of strandin was continued * in collaboration first with Dr. J. A. Meath, and later with Dr. S. Bogoch. In summary,[5] improvements in the methods of isolation permitted the preparation of strandin of higher purity and in higher yields than before. Conditions were found for the stepwise hydrolysis of strandin by a procedure in which different constituents were released gradually, leaving the rest of the strandin molecule as an undialyzable core. The chromogenic group was isolated and identified as ovine sialic acid. This and the finding by others [6] that gangliosides prepared according to the method of Klenk contained galactosamine in addition to sialic acid made it likely that gangliosides and strandin were closely related, if not identical, substances. In further confirmation of this possibility, strandin was found to contain both glucose and galactose, and its hexosamine was shown to be galactosamine. With the information thus gathered, it was possible to recognize some general features of the organization of the strandin molecule.

The Significance of the Combined Amino Acids Found in Strandin. Development of a General Method for the Quantitative Separation of Strandin from Lipide Mixtures.

The first phase of this study concerned itself with the significance of the combined amino acids found in strandin prepared by the partition-dialysis method. Two obvious possibilities had to be considered. One was that the combined amino acids were contaminants. The other was that they were, in fact, constituents of a strandin-like substance or substances and that the tissue contained at least two different types of strandin, one free of combined amino acids, and the other having amino acids among its constituents. The latter possibility was rendered plausible by the uniform failure of different attempts to separate the combined amino acids from the rest of the strandin preparation by solvent fractionation.

The partial solution of this question was a by-product of a continuing search for a procedure for the quantitative isolation of strandin. Since it was already known that, under proper conditions, strandin was extracted quantitatively from the tissue with 2:1 chloroform:methanol v/v,[7] the problem was to find a method for the quantitative separation of strandin from the lipide mixture present in chloroform-methanol tissue extracts. It was found that this could be accomplished by adding water to the extract in the proportion of 6.6 ml. of water per 100 ml. of extract, mixing the liquids, and letting the system stand overnight. Two clear phases separated; the upper phase (first upper phase) was collected, and to the lower phase was added the same amount of water as had been originally added to the extract. The mixture was handled as described above, and a second upper phase was obtained. It was found that the two upper phases contained over 95% of the browning reaction-giving substances in the tissue extract, and that the repetition of the procedure with the lower phase from the second distribution yielded upper phases containing only traces of browning reaction-giving substances. In summary, the first and second upper phases contained essentially all of the strandin from the tissue extract. In addition to strandin and small amounts of other lipides, they contained all the water-soluble, non-lipide substances from the original tissue extract, but these non-lipide contaminants could be eliminated quantitatively by dialysis. Thus, by combining the first and second upper phases and dialysing them exhaustively, it was possible to separate strandin quantitatively from the bulk of the lipides in the chloroform-methanol extract. This method was applicable to any lipide mixture, provided the proportions of chloroform, methanol and water used were adjusted to take into account the amount of

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water present in the tissue chloroform-methanol extracts.

Separation of "Peptide-Strandin."—In the course of the study of the chloroform-methanol-water procedure described above, it was found that the first upper phase yielded strandin richer in combined amino acids than the strandin yielded by the second upper phase. However, no further concentration of amino acids in any one fraction could be obtained by repeating the procedure with first upper phase strandin. Eventually, an amino acid-rich fraction was obtained by dissolving first upper phase strandin in 4-fold its weight of water and diluting the solution 50-fold with 9:1 methanol:chloroform, v/v. A clear solution results which, on standing overnight at −10°, yields a precipitate. The precipitate contains the bulk of the combined amino acids, and by repeating the procedure, a material was obtained which contained 18% combined amino acids. Shortage of material prevented attempts at further fractionation.

This "peptide-strandin" contained 5.5% N, <0.02% P and S, and <0.05% α-amino acid N. Hydrolysis by 6N HCl at 100° for 16 hours increased the value for α-amino acid N to 1.80%. The material was readily soluble in water as an undialyzable solute, and only sparingly soluble in organic solvents. It gave a browning reaction almost as intense as that of strandin and it yielded typical strandin strands. Its constituent amino acids were estimated both by starch column chromatography and by quantitative two-dimensional paper chromatography, which was kindly run by Dr. M. K. Gaitonde of our laboratory. There was good agreement between the results obtained by the two methods. The amino acids present were, in order of decreasing concentration, glutamic acid, aspartic acid, glycine, alanine, serine, lysine, leucine, arginine, threonine, valine, phenylalanine, γ-amino butyric acid, and traces of methionine. The analysis of the mother strandin showed that it contained the same amino acids as did peptide-strandin but in different molar ratios, i. e., some of the fractions discarded in the course of the preparation of peptide-strandin must have amino acid patterns different from that of peptide-strandin.

Development of a Simplified method for the Extraction of Strandin from the Tissue.

It was pointed out in the original report on strandin that, in spite of its high solubility in water, strandin could not be extracted from the tissue with water or with aqueous solutions. This suggested that, in the tissue, strandin was combined with a water-insoluble component, presumably a protein. In the extraction with chloroform-methanol, the postulated bond would be broken and strandin would be made available for extraction. In a series of experiments aimed both at gathering information on the postulated protein-strandin bond, and at finding a procedure for the extraction of strandin which would not require the use of chloroform, gray matter was homogenized at different temperatures with the following solvents, either singly or consecutively: ethyl ether, acetone, methanol and water. The amount of strandin in the different extracts was estimated by the chloroform-methanol-water method. It was found that none of these solvents or any combination of them extracted strandin at room temperature. Water extracts contained it in small amounts but the strandin that was present appeared simply to be due to a fine emulsion of all tissue components. On the other hand, hot methanol was found to extract strandin almost quantitatively. Furthermore, when the hot methanol extract was placed at 4°, strandin precipitated almost quantitatively. This constituted the basis for the following simple method for the isolation of strandin, which can be run on any scale that is technically feasible: Either gray matter or whole brain is homogenized with 5-fold its volume of methanol, and the homogenate is warmed to 60° and filtered while hot. The filtrate is placed at 4° overnight and the resulting precipitate is collected over a Büchner funnel. From the precipitate, strandin is isolated by the chloroform-methanol-water method and finally purified by exhaustive dialysis. Purification by the

chloroform-methanol-water method is repeated until the desired degree of purity is reached. P content is a reliable gauge of the degree of contamination of strandin prepared by this method. Usually, to prepare strandin with <0.1% P, the chloroform-methanol-water procedure must be repeated three times with extracts from gray matter, and up to five times for whole brain extracts.

By this procedure, the yield of crude strandin containing up to 1% P is about 3.5 grams per kilogram of gray matter, and half as much per kilogram of whole brain. The yield of strandin with <0.1% P would be about one-half these values. Of course, these yields do not reflect truly the efficiency of the extraction by hot methanol, since, because of the relatively low ratio of solvent to tissue, a substantial part of the extract is left with the tissue residue.

Properties and Composition of Strandin Prepared by Hot Methanol Extraction.—Strandin prepared by this method is a colorless or very light yellow, birefringent powder. It is readily soluble in water and insoluble, or scantily soluble, in organic solvents. On drying of its aqueous solutions, strands form less easily than with earlier, cruder preparations. Its electrophoretic and ultracentrifuge analyses, kindly run by Dr. Karl Schmidt of the Massachusetts General Hospital, Boston, show that it is homogeneous in the electrophoretic field; its ultracentrifuge analyses have been run in 0.1 M aqueous NaCl at 0.5%, 1.0%, 1.5% and 2.0% concentrations of strandin. In all cases, the preparation has been found to be homogeneous with the exception of a small, fast shoulder which amounted to <5% of the material. The sedimentation constant of the main component, which accounted for 95% of the sample, obtained in duplicate runs, has been as follows: *0.5%*, s12.5, 13.2; *1.0%*, s12.1, 12.8; *1.5%*, s11.5, 11.7; and *2.0%*, s11.1, 11.0. Aqueous solutions of strandin are not especially viscous and exhibit no birefringence of flow. These facts and the apparent independence of the sedimentation constant from concentration suggest that strandin in water solution is a globular particle. For a partial specific volume of 0.77-0.79, which was obtained with relatively cruder preparations, a minimum particle size of 250,000 can be computed.

Strandin prepared by this procedure is essentially free of amino acids (α-amino acid N, after hydrolysis: <0.02%) and contains only small amounts of phosphatides (P: <0.1%) and sulfatides (S: <0.05%). It contains 2.9% N; 24.0% neutral sugars, as galactose; 10.2% galactosamine; 30-31% sialic acid (chromogenic group) and about 30% fatty acid(s) and sphingosine (or a sphingosine-like substance). Its neutral equivalent is 1,150. By comparison with earlier preparations, it contains more N (2.9 as against 2.6%), more neutral sugars (24 as against 20-21%) and it has a lower neutral equivalent (1,150 as against 1,500). Strandin gives the following positive reactions: direct Ehrlich, Bial, diphenylamine, and tryptophane-$HClO_4$, with production of colored products qualitatively identical with those given by gangliosides.

Stepwise Hydrolysis of Strandin: Autohydrolysis.—From the very beginning of the study of strandin, a serious obstacle to progress in the elucidation of the chemical structure of strandin had been that any method of hydrolysis that had been attempted had resulted in the partial or total destruction of one or more of the constituents of strandin. Thus, because of the high resistance of strandin to alkali, the conditions for its effective alkaline hydrolysis resulted in the destruction of the constituent neutral sugars as soon as they were split off as free sugars. When acid was used, strandin was readily hydrolyzed but the massive production of humin-like material resulting from the destruction of the chromogenic group by the acid made further processing of the products of hydrolysis difficult. The solution of this impasse was the result of a systematic search for conditions under which strandin would be hydrolyzed without concomitant destruction of chromogenic group. It was found that

when an aqueous solution of strandin as free acid (i. e., strandin from which cations had been removed by treatment with cold aqueous HCl followed by exhaustive dialysis) was heated in a boiling water bath, it underwent partial hydrolysis, with the result that the major part of the chromogenic group was split off in a matter of minutes, without any substantial concomitant release of other constituents. Instead, the balance of the strandin constituents remained combined with one another in a number of undialyzable (i. e., large particle size) compounds. The chromogenic group that had been set free could easily be separated from the products of hydrolysis by a number of different procedures, such as distribution between 50% aqueous methanol and chloroform, precipitation from aqueous methanol, differential centrifugation or dialysis. In the course of the autohydrolysis of strandin, there was a sudden marked increase in viscosity at the time of the release of the bulk of the chromogenic group, presumably because of the breaking up of the large globular particle into asymmetrical fragments. This increase in viscosity, which takes place in a fraction of a minute, permits the accurate timing of the interruption of the reaction in any one run.

After removal of the chromogenic group which had been set free, the balance of the strandin molecule can be submitted to hydrolysis without interference from the browning reaction. Different hydrolytic agents can be used, but 0.09N HCl at 100° has been found to be specially suitable for the gradual release of constituents over a period of many hours, with the rest of the strandin molecule still being left as an undialyzable core. Thus, at any point, the released constituents can be separated by dialysis. The core can finally be hydrolyzed completely by 6N HCl at 100°.

Study of the Products of Stepwise Hydrolysis.—In the particular hydrolysis to be discussed here, the actual conditions used were the following: A 9% aqueous solution of strandin as free acid was heated at 100° until marked viscosity developed, which took 18 minutes. The solution was cooled and dialyzed, and the dialysate collected (*dialysate A*). The undialyzable fraction was treated with 0.09N HCl at 100° for one hour and then cooled and dialyzed, and the dialysate collected (*dialysate B*). The undialyzable fraction was dissolved in 6N HCl at room temperature, and the solution placed in a boiling water bath for 4 minutes. This was 30 seconds beyond the formation of a massive precipitate. The solution was rapidly cooled in ice water and dialyzed. *Dialysate C* and a *final undialyzable fraction* were obtained.

Dialysate A: Identification of the Chromogenic Group as Ovine Sialic Acid.—This fraction accounted for 21% of the weight and 66% of the chromogenic group of the original strandin. It contained small amounts of neutral sugars and hexosamines and it consisted otherwise of a material which could be divided into about equal amounts of a *methanol-insoluble* and a *methanol-soluble* fraction.

The *methanol-insoluble* fraction was a crystalline white or slightly pinkish powder, consisting of small needles. *Neutral equivalent*, 343; *N*, 4.3%; NH_2-*N*, <0.05%; *browning reaction*, 2.65-fold that of strandin; *reducing power*, 50% that of galactose. It was shown to be identical with or closely related to Blix's sialic acid[6] on the basis of (i) its elementary composition; (ii) the presence, for each $-COOH$, of one reducing group, 1 N atom, and one tetraol; (iii) its decarboxylation by 3.5N HCl at 100°; (iv) its positive direct Ehrlich and Bial reactions, with formation of products with spectra qualitatively and quantitatively similar to those formed by sialic acid; and, (v) its x-ray diffraction pattern, kindly run by Professor G. Blix, which was undistinguishable from that of ovine sialic acid.

The *methanol-soluble* fraction was an amorphous, reddish hygroscopic powder of composition very close to that of the crystalline, methanol-insoluble sialic acid. Its absorption spectrum exhibited a shoulder at 270 millimicrons which sialic acid did not show. A study by ultraviolet spectrometry

of the procedure of autohydrolysis showed (*a*) that strandin, either as a salt or as a free acid, had no special absorption in the ultraviolet range; (*b*) that it developed a shoulder at 270 millimicrons during autohydrolysis; (*c*) that crystalline sialic acid, which showed no special absorption in the ultraviolet range, developed a shoulder at 270 millimicrons on heating its aqueous solution at 100° for 30 minutes; and (*d*) that the spectra developed by strandin as a free acid, and by sialic acid on heating in aqueous solution were identical with each other and with the spectrum of the methanol-soluble, amorphous fraction. All these observations suggested that the methanol-soluble fraction was a product of breakdown of sialic acid during autohydrolysis.

Dialysate B.—This fraction accounted for about 17% of the weight of strandin. By analysis, it appeared to contain 40% sialic acid, 15% galactosamine, and 22% neutral sugars, as galactose, the balance of the fraction presumably being breakdown products of sialic acid. The only neutral sugar that could be demonstrated was galactose. Most of the galactose and galactosamine was combined as oligosaccharides.

Dialysate C.—It accounted for 31% of the weight of strandin. It contained 73% neutral sugars and 17% galactosamine, mostly combined as oligosaccharides. On further hydrolysis, both galactose and glucose were shown to be present.

Final Undialyzable Fraction.—It accounted for 33% of the weight of strandin. It consisted mostly of fatty acid(s) and sphingosine (or a sphingosine-like substance), apparently in equimolar amounts with about 6% combined carbohydrate which, on further hydrolysis, proved to be only glucose. Only traces of galactose and of galactosamine were found in the final hydrolysate.

The Chemical Structure of Strandin.—While the complete elucidation of the structure of strandin will require much more work, the information already gathered has established a few points and suggested a number of possibilities. They are: (*a*) Sialic acid is combined with the rest of the strandin molecule through its reducing group. That this is the case is shown by the lack of reducing power of strandin, i. e., the reducing group of sialic acid is not free; by the gradual appearance of reducing power in the course of autohydrolysis, at a rate and of a magnitude consistent with its being due to the release of free sialic acid; and by the observation that the undialyzable fraction after autohydrolysis shows no free reducing groups. (*b*) The ceramide moiety (fatty acid-sphingosine) is combined mainly, if not exclusively, with glucose. This glucose, in the final undialyzable fraction, is the only carbohydrate combined with the ceramide moiety. Also, Bogoch,[8] presumably starting from a preparation identical with our own, has obtained a glucocerebroside in which glucose and ceramide are present in equimolar amounts. (*c*) The demonstration of large amounts of oligosaccharides among the products of stepwise hydrolysis of strandin shows that glucose, galactose and galactosamine are combined with each other. (*d*) The order of release of the different carbohydrate constituents, which shows that galactosamine and galactose are released first, and glucose released later, suggests, but does not establish, that sialic acid is combined with either galactosamine or galactose.

Any speculation on the structure of strandin beyond the points discussed above would be idle in the sense that it would lack any base of observed facts. There is, however, one point which should be discussed. This is the question of the molecular size of strandin (and, by inference, of gangliosides). The physical properties of strandin leave no doubt that, in aqueous solution, it exhibits a particle size of $>250{,}000$. On the basis of the least abundant constituent (glucose or galactosamine), a minimal molecular weight of 2,000 to 3,000 is indicated. Hence, if the particle size represents molecular size, strandin must be constituted by a considerable number of repeating units, and the problem of the structure of strandin involves not only the elucidation of the structure of the basic unit, but also the eluci-

dation of the nature of the bond or bonds between the repeating units. If this is the case, a polysaccharide structure, to which sialic acid and ceramide groups would be linked as side chains, comes naturally to mind. On the other hand, if it is postulated that the particle size indicates only aggregation of much smaller, single molecules, it is natural to assume that the structure of strandin is that of a complex cerebroside.

The evidence thus far available does not permit any conclusion as to the actual molecular size of strandin. This all-important question can only be answered by further work.

The Relationship Between Strandin and Klenk's Gangliosides.—It is obvious from the foregoing that there are many similarities between strandin and the gangliosides isolated by Klenk. Thus, they both have as constituents, sialic acid, galactose, glucose, galactosamine, fatty acids and sphingosine in approximately the same molar ratios and they both give a number of positive reactions, with production of qualitatively identical colored products. They are both soluble in water as undialyzable solutes. On the other hand, gangliosides have been crystallized as spherules or rosettes, a crystalline form that we have never observed with strandin. Also, strandin is reported to contain a larger concentration of sialic acid than gangliosides. Finally, strandin is obtained from brain in much higher yields, and appears to be present in immature brain and in white matter in vastly larger amounts than are gangliosides.

Clearly, until the structures of both these substances or groups of substances have been fully elucidated, their exact relationship cannot be known. However, a consideration of the similarities and differences enumerated above, weight the balance greatly in favor of a very close connection between the two, in the sense that the similarities are much more positive than the differences. Indeed, many of the differences can be explained as being due to the particular methods used. Thus, in the estimation of sialic acid, different standards have been used by different groups of investigators, and the values reported may not be comparable from group to group.

In summary, it is proposed that strandin be designated as ganglioside strandin to emphasize its close connection with the gangliosides of Klenk. However, since strandin refers to a preparation which is obtained with a high degree of reproducibility, it is desirable that the use of the term be continued, until its exact relation to other ganglioside preparations has been established.

Analysis of Pathological Material.—Several human brains with overt neuropathology have recently become available to us for analysis and among these were brains from two cases diagnosed as amaurotic familial idiocy. One was a case of infantile Tay-Sachs' disease †; the other, the juvenile or Bielschowsky type of the disease.‡ In each case, gray and white matter were dissected and treated separately. A weighed amount of tissue was homogenized with chloroform-methanol, 2:1 v/v and the homogenate filtered quantitatively through a pre-weighed sintered glass filter. Total tissue dry weight was obtained as the sum of the weight of the residue remaining on the filter and the weight of solutes in the chloroform-methanol filtrate. The residue on the filter was washed exhaustively with water and acetone and the weight of the remaining residue represented total tissue proteins minus proteolipide protein. The chloroform-methanol filtrate contained all the lipides and proteolipides and some non-lipide contaminants. The non-lipide contaminants were removed by washing the extract once with one-fifth its volume of water and twice with $CHCl_3$:CH_3OH:H_2O 5:48:47 v/v/v containing KCl as described by Folch, Lees and Sloane Stanley.[9] The washed lipide extract was analyzed for total phosphatides, alkali-labile phosphorus, phosphatidyl serine, cerebro-

† Obtained from Dr. Alan Crocker of Children's Medical Center, Boston.

‡ Obtained from Drs. E. P. Richardson and G. Lyon of the Massachusetts General Hospital, Boston.

*Composition of Gray and White Matter in Two Cases of Amaurotic Family Idiocy (AFI)**

	Infantile AFI (2 yr., 10 mo.)		Juvenile AFI (4 yr., 6 mo.)		Normal † (2-5 yr.)	
	gray	white	gray	white	gray	white
Total solids	17.9	16.5	13.5	23.0	13.0	25.7
Water	82.1	81.5	86.5	77.0	87.0	74.3
Total lipides (minus strandin)	5.58	4.80	4.60	12.38	4.63	14.1
$CHCl_3$:CH_3OH insol. residue	8.15	9.60	7.18	6.92	--	--
Phosphatides	3.0	2.42	2.63	5.54	2.68	6.25
Phosphatidyl serine	0.31	0.25	0.27	0.93	--	--
Sphingomyelin	0.59	0.51	0.47	1.14	0.48	1.6
Cerebrosides	0.42	1.21	0.19	1.83	0.31	3.34
Proteolipide protein	0.62	0.53	0.59	1.93	--	--
Strandin (gangliosides)	1.46	1.05	0.21	0.16	--	--

* All results expressed as grams per 100 grams fresh tissue.

† Data calculated from Brante; both total lipide and cerebroside values probably include strandin.

sides, and proteolipide protein by appropriate methods. The combined upper phases were concentrated, dialyzed and analyzed for the brain ganglioside, strandin, by the browning reaction given by its chromogenic group.

The Table shows the results of the analyses of brain gray and white matter from the two different types of Tay-Sachs' disease. Values for gray and white matter from normal brains of a similar age group have been calculated from the data of Brante [10] and are presented for comparison. It can be seen that, whereas both the total solids and the lipides (excluding strandin) are markedly decreased in the white matter in the infantile form of the disease, only minimal changes are found in the juvenile form. This observation is in agreement with the histological picture which showed marked demyelination in the former and only minimal white matter destruction in the latter. The decrease in total lipides cannot be attributed to any specific lipide fraction, all fractions showing similar proportional decreases. There is, however, a very striking increase in the amount of strandin in the white matter in the infantile Tay-Sachs' disease. Normally, this component is present in white matter in relatively low concentration amounting to only about one-tenth that found in gray matter. Unfortunately, no quantitative data are available for strandin in the 2-5 year old age group, but the values for normal human adult gray and white matter are 0.7 and 0.07 grams per 100 grams fresh tissue respectively. The gray matter shows no decrease in total solids in either form of the disease; there is, in fact, an increase in total solids in the infantile form which can be attributed mainly to a marked increase in strandin. The juvenile form, on the contrary, shows no increase in strandin and, actually, the values for this component appear to be slightly low.

A comparison of these two cases thus shows that, both histologically and chemically, the changes in the white matter in infantile Tay-Sachs' disease are more marked than in the juvenile form of the disease; this comparison applies to the increase in strandin as well as to the decrease in other chemical components. The results, however, must be interpreted with caution since they are based on only one case of each type. Furthermore, we are limited by our inadequate knowledge of the specific areas being analyzed and of the chemical composition of the developing human brain in general.

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Some Observations on the Mucolipids of Normal and Tay-Sachs' Disease Brain Tissue

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We have recently proposed the group designation of "mucolipid" [1] for the complex lipid polymers that combine characteristics of the cerebrosides with others usually assigned to the mucoids. This term, which has since enjoyed wide adoption, favors concomitant recognition of the existence of a varied family of related but unique substances, soluble in both water and organic solvents, and having, as constituents, fatty acid, sphingosine or a similar base, hexose, and also N-acetyl hexosamine, often amino acids and, most significantly, sialic acid or a related substance. Lipids of this type were encountered 23 years ago by Landsteiner and Levene [2]; in succeeding years, they have been uncovered in spleen,[3] normal brain,[4] Niemann-Pick brain [5] and, in dramatic superabundance in human brain tissue in advanced stages of infantile amaurotic idiocy (Tay-Sachs' disease).

Our original investigations on the mucolipids of brain were prompted by several unique observations. It was found that a preparation obtained by extensive purification of a crude fraction isolated from beef brain by the ingenious partition-dialysis procedure of Folch and collaborators [6] contained a considerably higher proportion of sialic acid than had been recognized at the time.[1,10] This preparation was found also to differ, subtly but convincingly, from ganglioside preparations, both in a marked inhibitory action on the agglutination of chicken erythrocytes by influenza virus [1] and in the ready susceptibility of its sialic acid moiety to hydrolytic release by the "receptor destroying" enzyme of V. cholerae. Details of the isolation and characterization of a physically homogeneous beef brain mucolipid from this fraction have been presented elsewhere.[7] The lipid can be described as a highly polymerized ampholytic compound consisting of different quantities, in descending order, of galactose, sialic acid, fatty acid and sphingosine in equal proportions, amino acid, N-acetylgalactosamine, and glucose. The molar proportions of the constituents (listed in Table 1) make it obvious that one is dealing with an extremely complex substance for which, for the time being, no simple repeating unit should be put forward. This lipid appears to be one of the more complex representatives of its class.

One valid approach to an understanding and potential control of infantile amaurotic

TABLE 1.—*Composition of Beef Mucolipid; Balance* *

Component	Weight Distribution	Molar Distribution	No. of Component Molecules per Mucolipid Molecule
	per cent of mucolipid	Micromole per mg. mucolipid	
Fatty acid	20.6	0.56	101
Sphingosine	16.5	0.55	99
Hexose †	23.0	1.3	234
N-Acetyl galactosamine	4.5	0.20	36
Sialic acid	26.0	0.84	151
Amino acid	4.4	0.32	58
Unbound water	2.6		
Total	97.6		
Correction ‡	6.3		
Corrected total	91.3		

* The molecular weight of the mucolipid is taken as 180,000; for fatty acid the molecular weight of lignoceric acid, 368.6, was used; the average molecular weight of amino acid was computed from distribution studies similar to those summarized in Ref. 7.

† The ratio of galactose to glucose is approximately 8:1.

‡ This correction allows for water eliminated by bonding.

From the Cell Chemistry Laboratory, Department of Biochemistry, College of Physicians and Surgeons, Columbia University.

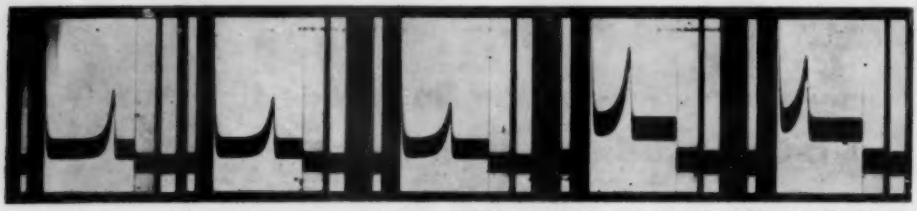


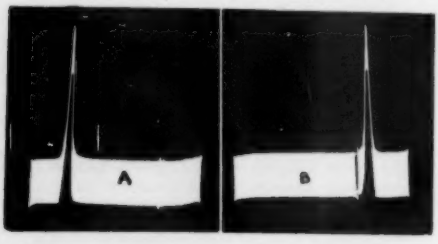
Fig. 1.—Sedimentation pattern of the purified Tay-Sachs ganglioside, 1% unbuffered aqueous solution, sedimentation constant = 7.1 S.

idiocy lies in a careful comparison of the mucolipid found in human brain tissue under normal conditions with that in the diseased state, in which so great a quantity finally accumulates as to occasion an enormous swelling of the neural and eventually the glial cells, with a simultaneous nearly complete displacement of cerebroside. The present study outlines the preliminary findings of such a comparison.

Isolation of Human Brain Mucolipids

Klenk [8] was the first to isolate and describe the brain mucolipid which accumulates in infantile amaurotic idiocy; he named the substance "ganglioside" [9] after its occurrence in ganglion cells. For the present study, the procedure elaborated by Klenk for the isolation of ganglioside was modified to allow the use solely of neutral solvents and a minimal exposure of the tissue extracts to elevated temperature. The finely divided whole brain was treated with several changes of a vast excess of cold acetone followed by extraction of the dried, finely pulverized tissue for four 5-minute periods with boiling anhydrous methanol in the ratio (w/v, dried tissue to methanol) of 1:3. The extracts were filtered hot, combined, and allowed to stand overnight at 4°. The resulting "protagon" precipitate (approximately 3 grams per 100 grams wet weight of tissue) was reprecipitated from chloroform-methanol (2:1, v/v) and twice again from methanol. The resulting material, amounting to somewhat more than ½ of the crude starting material was homogeneous in the ultracentrifuge (Fig. 1) and in the Tiselius apparatus (Fig. 2). Its composition will be presented later. The mucolipid of normal child's brain was isolated and purified by the procedure employed for beef brain.[7] This consisted essentially of extraction of the fresh tissue in the cold with chloroform-methanol, followed by dialysis of the filtered extract against water (partition dialysis). The resulting aqueous upper phase containing the mucolipid was concentrated, redialyzed, and lyophilized. The crude mucolipid residue was dissociated with $CaCl_2$ and purified by successive repartition between chloroform and water with the aid of methanol, followed by counter-current distribution between the two phases formed on mixing carbon tetrachloride, methanol, and water (1.5:1.5:0.5, v/v/v).

Fig. 2.—Electrophoretic pattern (*A*, initial; *B*, final) of the purified Tay-Sachs ganglioside, 1% solution, pH 5.1, $\mu = 8.6 \times 10^{-5}$ cm²/volt sec.



Brain tissue from a Tay-Sachs case, frozen soon after removal and stored several days in the frozen state, was subjected to the same protocol, whereupon an interesting phenomenon presented itself. Cold chloroform-methanol extraction left no detectable residue of ganglioside in the tissue. Yet, examination of the upper aqueous phase following partition dialysis of the extract revealed a quantity of mucolipid only slightly greater than that found in

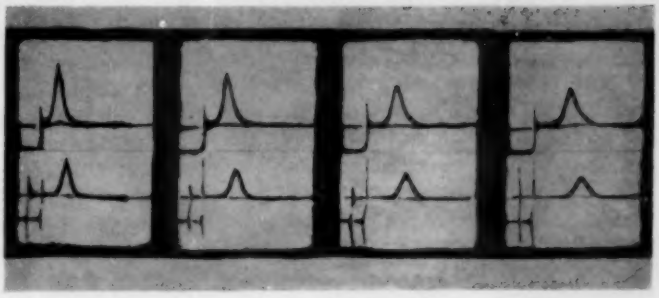


Fig. 3. — Comparison under identical conditions in a split sector cell of the sedimentation patterns of the "normal" mucolipids isolated both from the brain of a Tay-Sachs case (upper pattern) and a normal control (lower pattern). The initial boundaries have been alined to facilitate comparison.

normal brain. This material was very similar to that obtained from normal brain with respect to composition. The two lipids were indistinguishable in the ultracentrifuge (Fig. 3). Examination of the lower phase plus interfacial material showed that the bulk of the gangliosides were contained in this fraction. Since the latter also contains the majority of other tissue lipids, isolation of the gangliosides therefrom has not yet been effected. It appears, therefore, that Tay-Sachs ganglioside is not amenable to extraction by the procedure which successfully produces the mucolipid of normal brain. On the other hand, isolation of the latter by hot methanol extraction of the acetone-dried brain tissue is impractical because of the great amounts of cerebroside which are extracted thereby and serve to mask the relatively minor mucolipid fraction. These observations on extractability point to a physical difference between the two lipids in question, a difference which is apparently mirrored in their relative behavior in countercurrent distribution (Fig. 4). Comparison is made, therefore, between the ganglioside isolated from Tay-Sachs brain by the simplified extraction procedure outlined above and the mucolipid isolated from normal child's brain by the partition-dialysis protocol.

Comparison Between the Mucolipids of Normal and Tay-Sachs Brain

Methodical details for the analyses in this section are presented elsewhere.[7] A salient

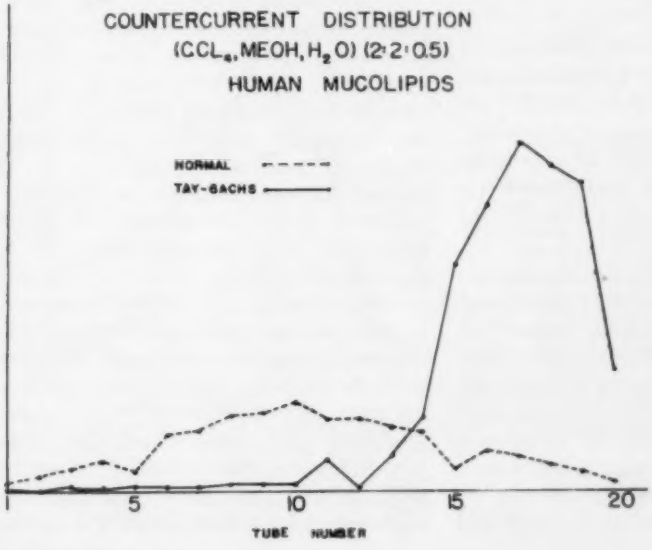


Fig. 4. — Countercurrent distribution of human mucolipids between the phases formed on mixing carbon tetrachloride, methanol, and water.

TABLE 2.—*A Comparison of Some Properties of Brain Ganglioside from a Tay-Sachs Case with Those of the Mucolipid from Normal Human Brain*

Property	Tay-Sachs Ganglioside	Normal Human Mucolipid
% Sialic acid	24	21
% Hexose	32	33
Glucose: galactose	1:7	1:8
% N-acetyl galactosamine	8	4.4
Amino acids	Trace	Numerous present
Sedimentation constant	7.15	8.55
R_F (countercurrent)	0.85	0.50
Metachromatic effect	Marked	Slight
Sialic acid release (RDE)	7%	51%
Hemagglutination inhibition	Inactive	Active

feature of the comparison of the properties of the mucolipids from normal and Tay-Sachs brain (Table 2) is the essential similarity, component-wise, of these substances. The two lipids are not readily distinguishable on the basis of hexose, N-acetyl galactosamine, or sialic acid content. The outstanding point of contrast here is the presence of a peptide moiety in the material from normal brain and its absence in that from the Tay-Sachs case. The nature of this peptide is under investigation at present. A study to determine the component amino acids in a manner analogous to that undertaken for the beef brain mucolipid [7] is now in progress.

Aside from an apparent difference in particle size as evidenced by sedimentation values (7.1 S for ganglioside and 8.5 S for normal mucolipid), the observed differences, although relatively dramatic in manifestation, are rather subtle in origin and hence difficult to evaluate. They will be treated individually below.

Susceptibility to RDE.—For this experiment, 1.0 mg. quantities of lipid and 0.5 mg. of RDE ("receptor destroying enzyme"), a lyophilized filtrate of V. cholerae (kindly supplied by Dr. R. H. Barnes of Sharp and Dohme, Inc.) in a 3-ml. lipped test tube were dissolved in 0.3 ml. of 0.05 M acetate, pH 6.1, the mouth of the tube fitted with a cellophane membrane snugly secured by several turns of a small rubber band, and the end of the tube suspended in 3 ml. of buffer, the whole being incubated with gentle shaking for 16 hours at 31°. Under these conditions, enzymatically released sialic acid dialyzes into the larger outer volume of buffer, thus favoring further hydrolysis. 51% of the sialic acid of the mucolipid from normal brain was rendered dialyzable while, under identical conditions, less than 10% of the sialic acid of the ganglioside from Tay-Sachs brain was split. This resistance of the Tay-Sachs material to enzymatic attack, which was pointed out previously,[11] is surprising in view of a sialic acid content practically identical with that in the normal mucolipid, the identity of the sialic acid fractions of both lipids as shown by infra-red analysis, and the similar behavior of both lipids towards dilute acid hydrolysis. It may be presented as a working hypothesis that susceptibility to enzymatic attack depends not only on the sialic acid moiety but also on the presence of the proper peptide moiety. Such a working hypothesis may be further extended towards an understanding of the metabolic defect in Tay-Sachs' disease in which it may be possible that a biologically useless mucolipid, lacking the peptide fraction, accumulates.

Metachromasia.—Although long recognized,[11] the phenomenon of metachromasia, characterized by a change in the color of solutions of many basic dyes in contact with certain histological elements, still is not completely understood. The color change is based on a diminution of the original (orthochromatic or α) absorption maximum of the dye accompanied by the appearance of a new maximum (β) of approximately 30 mμ shorter wavelength, as well as other less well defined (γ) maxima, at still shorter wavelengths. Substances known to produce metachromatic shifts are, generally speaking, polysaccharides containing acidic functional groups.[12] It has been reasonably well substantiated that, while metachromasia is due to binding of the dye by the polymer, it is not [13] caused by polymerization of the former on the surface

of the latter.[14] It has also been shown that a direct relationship exists between the magnitude of the shift and the number and dissociation characteristics of the acidic radicals on the polymer; and that direct combination of the acidic group of the polymer and the basic group of the dye occurs.[13] While a polymer of some sort is required for the metachromatic effect, the effect is not related to the degree of polymerization, but depends in some way on the solubility characteristics of the dye-substrate complex. The interesting observation by Feyrter[15] that the nerve cells in Tay-Sachs' disease are strongly metachromatic prompted a study of the in vitro metachromatic behavior of Tay-Sachs ganglioside and a comparison with the normal brain mucolipid.

1.0 mg. of (*a*) Tay-Sachs ganglioside, (*b*) normal beef brain ganglioside, (*c*) crude mucolipid, and (*d*) highly purified mucolipid, dissolved in 10.0 ml. of 0.05 M HCOOH buffer pH 3.7, were mixed with 1.0 ml. of a 0.1% (w/v) solution of toluidine blue in the same buffer. The results are shown graphically in Figure 5. It may be seen that both the Tay-Sachs and beef ganglioside are strongly metachromatic, inducing a great reduction of the α- and the appearance of a well-defined β-maximum. The mucolipid from normal brain, on the other hand, exhibits only a slight metachromatic effect. Interpretation of this phenomenon is difficult, although it may be postulated that the observed differences reflect, in some way, a difference in structure.

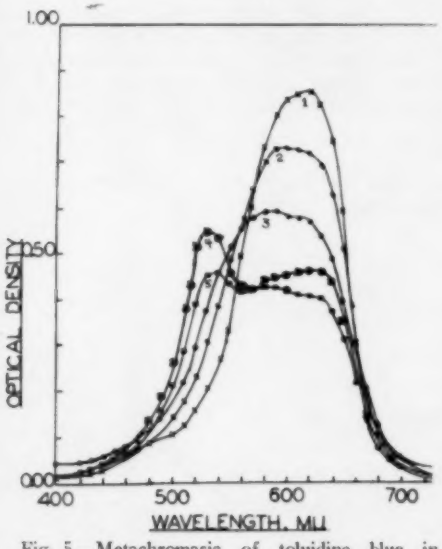


Fig. 5.—Metachromasia of toluidine blue in formate buffer, pH 3.9, induced by various mucolipid preparations. 1. dye alone; 2. purified mucolipid from normal brain; 3. crude mucolipid from normal brain; 4. ganglioside from Tay-Sachs brain; 5. ganglioside from normal beef brain.

Comment

As has been pointed out previously,[7] it is unfortunately not yet possible to discuss meaningfully the relationships, generic or specific, functional or metabolic, of the various forms in which cerebrosides occur in nervous tissue. Although the available figures[16] are not easily comparable, it would seem that the bulk of cerebrosides occur in normal brain tissue as monomers. In Tay-Sachs' disease, the reverse apparently holds: there is a great increase in polymeric mucolipid content with a severe concurrent diminution of monomeric cerebroside. While the studies here presented are admittedly fragmentary and still in a rather tentative state, it is hoped that a start has been made towards an understanding of the etiology of infantile amaurotic idiocy from a biochemical point of view.

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Neuraminic (Sialic) Acid Studies of Biological Fluids in Amaurotic Family Idiocy and Related Disorders

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In a recent review article on the serum glycoproteins, Winzler [1] called neuraminic acid (NA), or sialic acid (SA), "a component which has not yet been characterized chemically." He then added the prophetic statement, "It appears likely that the chemistry and distribution of this substance will be a topic of considerable interest in the future." The large number of papers which have appeared in the literature in the last few years dealing with the chemical structure of NA and its widespread occurrence as a constituent of the glycolipids and glycoproteins of many tissues and biologic fluids of animal origin has confirmed this prophecy. These publications indicate that this complex amino acid-sugar plays an important, if as yet undetermined, role in animal physiology.[2]

The structure of the "free" neuraminic acid (FNA) and some of its variously substituted derivatives (known collectively as "sialic acids") which have been isolated from biological material are illustrated in Figure 1. The chemical structures shown in this figure are those proposed by Gottschalk and co-workers [3,4] and which have been verified by means of the enzymatic synthesis of N-acetyl neuraminic acid (NANA) by Cornforth et al.[5,6] The NANA, whose structural formula is given in Figure 2, appears to be the NA derivative present as an integral part of most glycoproteins and glycolipids of serum, urine, ovomucin and other biological fluids and tissues.[3] Comparison of synthetic NANA with that isolated from biological material show them to be identical with

From the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn.

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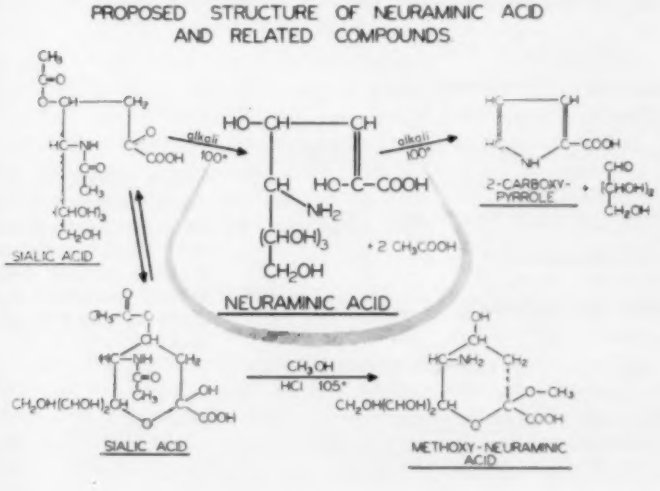

Fig. 1. — The chemical constitution of free neuraminic acid and some of its related compounds as proposed by Gottschalk.[3,4]

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The structure of the "free" neuraminic acid (FNA) and some of its variously substituted derivatives (known collectively as "sialic acids") which have been isolated from biological material are illustrated in Figure 1. The chemical structures shown in this figure are those proposed by Gottschalk and co-workers [3,4] and which have been verified by means of the enzymatic synthesis of N-acetyl neuraminic acid (NANA) by Cornforth et al.[5,6] The NANA, whose structural formula is given in Figure 2, appears to be the NA derivative present as an integral part of most glycoproteins and glycolipids of serum, urine, ovomucin and other biological fluids and tissues.[3] Comparison of synthetic NANA with that isolated from biological material show them to be identical with

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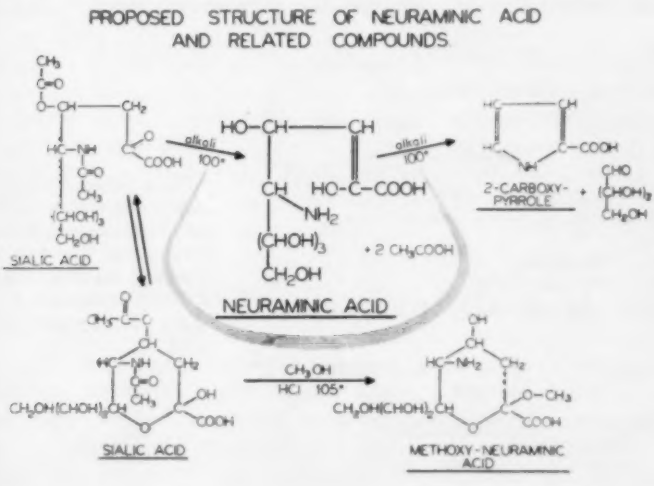
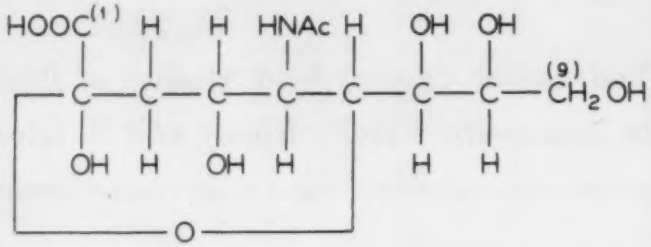


Fig. 1. — The chemical constitution of free neuraminic acid and some of its related compounds as proposed by Gottschalk.[3,4]



N - ACETYLNEURAMINIC ACID

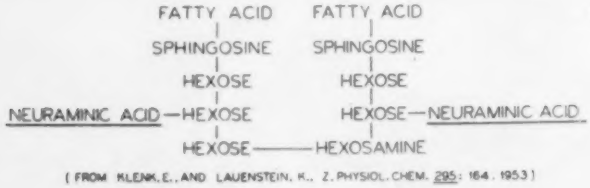
(From Gottschalk, A. "The Influenza Virus Neuraminidase" Nature 181, 377, 1958)

Fig. 2.—The structure of N-acetyl-neuraminic acid which is the derivative most commonly found in biological fluids and tissues.

respect to a large number of physical and chemical criteria.[5,6]

The FNA shown in Figure 1 is an α-keto, γ-amino acid sugar which has too many reactive groups to be found in this form in nature. Heating the FNA with alkali yields the 2-carboxy-pyrrole which was isolated from a split product formed by the action of the influenza virus enzyme on mucoproteins.[7] The diacetyl neuraminic acid derivative, is probably the type of "sialic acid" first isolated by Blix[8] from bovine submaxillary gland mucin and the methoxy derivative of neuraminic acid is that isolated by Klenk[9] as a cleavage product of human brain gangliosides. Substitution of H atoms by acetyl, methyl, glycolyl or other groups in the α, γ or δ positions on the -N- or -O- atoms gives a number of naturally occurring derivatives of NA which are known collectively as "sialic acid."[10] Such structures may be representative of their occurrence as a fundamental part of more complex biological structures such as glycoproteins and glycolipids.

The presence of NA derivatives as part of the complex ganglioside lipids from **brain gray matter is shown** in Figure 3 as reproduced from a paper by Klenk.[11] Recent publications by Bogoch[12,13] indicate that the sialic acid is linked through galactosamine to form a monomeric repeating unit having a molecular weight of 3,314 and that the gangliosides found in brain



(FROM KLENK, E., AND LAUENSTEIN, K., Z. PHYSIOL. CHEM. 295: 164, 1953)

Fig. 3.—Proposed structure of gangliosides present in animal brain tissue (after Klenk[11]) and some biological material from which neuraminic (or sialic) acid has been isolated.

NEURAMINIC OR "SIALIC" ACID FOUND IN :

1. BEEF SUBMAXILLARY MUCIN
2. BOVINE COLOSTRUM
3. RED CELL STROMA LIPIDS
4. MILK
5. URINARY AND SERUM GLYCOPROTEINS
   a. MUCOPROTEINS
   b. MUCOIDS (OROSOMUCOID)

tissue represent high molecular weight polymers of such monomers. Linkage of the NA to hexosamine, or hexose, is through its glycosidic reducing group to the rest of the glycolipid or glycoprotein.[13,14] Its position in the complex is such that at pH 1.0 and 80° C, almost all the sialic acid, and only the sialic acid, is released.[14] By means of acid hydrolysis to free the conjugated sialic acids, and by then applying distinctive color reactions [1,15] the occurrence of NA derivatives has been demonstrated in a wide variety of biological materials including red cell stroma lipids,[16] bovine colostrum,[17] milk,[18] urinary glycoproteins,[19,20] serum glycoproteins,[21,22] etc.

It was the reported presence of NA derivatives in blood serum [23,25] and cerebrospinal fluid [26,27] that motivated our own investigations into the possible relationship between the increase of the NA-rich gangliosides in the cerebral tissue of amaurotic family idiocy (AFI) and the levels of such substances in the serum and CSF of this disease. It was believed that such correlative studies might be useful in the diagnosis of AFI [28] and provide some clearer insight into the pathogenesis of neurological diseases characterized by disturbed lipid metabolism.[29] However, we were cognizant of the possibility that there need be no direct connection between the metabolic participation of a substance when it is part of a large tissue complex, and the same substance when it circulates in the various body fluids as a part of a protein bound complex (e. g., iron in the hemoglobin molecule and iron as part of the iron-binding globulin). The present paper summarizes the work conducted on this problem in this laboratory over the past three years.

Experimental

A. Material and Methods.—The distribution of the serum protein fractions was obtained by means of the Tiselius moving boundary method employing a technique previously published from this laboratory.[30] The total protein of both serum and the CSF was determined with the biuret method [31] using a standard serum protein solution which was checked with the micro-Kjeldahl procedure. The distribution of NA among the various serum protein fractions was determined by means of preparative starch block electrophoresis using the technique described by Kunkel [32] but employing silver-silver chloride electrodes which would permit runs to continue for 48 hours or longer without contamination of the starch block by electrode by-products. The details of this method, as well as those for the quantitative determination of the total neuraminic acid (TNA) content of serum and of its globulin fraction (GNA) using the relatively specific colorimetric diphenylamine (DPA) reaction have already been published.[33,34] A similar procedure for the quantitative determination of TNA, protein-bound neuraminic acid (PBNA) and "free" neuraminic acid (FNA) suitable for small samples of CSF has recently been developed in this laboratory.[35] The NA procedures for both serum and CSF have been applied to a large number of specimens derived from patients with various neurological diseases with special emphasis on AFI and related lipidoses as well as to patients with non-neurological disorders and to presumed normal control groups. The data obtained were then evaluated by means of techniques of group statistics.

B. Electrophoretic and Chemical Studies of the Serum Protein Neuraminic Distribution.—Since many of the isolated serum glycoproteins have been found to migrate with an electrophoretic mobility of the alpha-2 globulins in barbital buffer at pH 8.6, electrophoretic studies of the protein distribution in AFI sera were performed. The results obtained with the Tiselius moving boundary method for sera from patients with AFI, as compared to normal children, are shown in Figure 4. This figure and the accompanying table summarize the results obtained in previous publications from this laboratory.[34,36] These data show a significant decrease in the albumin fraction

SERUM ELECTROPHORESIS IN TAY-SACHS' DISEASE

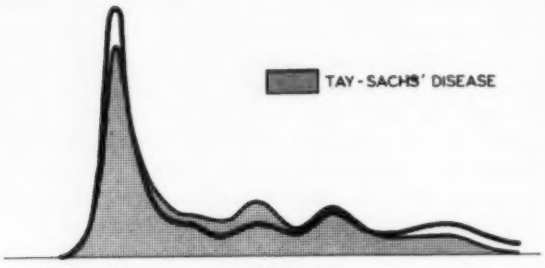


Fig. 4.—The serum protein electrophoretic pattern in amaurotic family idiocy (Tay-Sachs' disease) as compared with that obtained for normal serum. (From Volk, et al., *J. Lab. & Clin. Med.* 50:26-35, 1957.)

	Albumin gm%	Globulins gm%			
		alpha-1	alpha-2	beta	gamma
TAY-SACHS' DISEASE	3.70	0.50	0.98	0.96	0.65
NORMAL	4.07	0.48	0.66	0.96	1.01
P	<.01	.40	<.01	-	<.01

($p<.01$) which is to be expected with any severe chronic disease. There is, however, a significant increase in the alpha-2 globulin, the fraction containing most of the glycoproteins as well as some of the lipoproteins.[37] An interesting and somewhat unexpected finding was the significant decrease in the gamma globulin fraction.

The distribution of NA-protein conjugates in AFI and normal sera relative to their electrophoretic protein fractions was then investigated more minutely by means of the preparative starch block electrophoretic technique of Kunkel.[32] Six specimens of AFI sera were run with this method in comparison to six normal sera. After completion of the electrophoretic run, the starch block was cut into 30 sections and the eluted protein from each cut was analyzed for its protein content by the Lowry[38] method and for its NA content with the DPA reaction.[33] A typical set of results for one normal serum and one AFI patient's serum, superimposed on a normal serum protein electrophoretic pattern, is illustrated in Figure 5. It can be seen from this graph that NA-containing proteins are distributed throughout the spectrum of the electrophoretic protein fractions. However, in agreement with other investigators,[1] the NA-rich protein fractions are those associated with proteins having an electrophoretic mobility of alpha- or beta-globulins in spite of the fact that about 60% of the TP is present in the albumin fraction. In the AFI sera, as contrasted to normal controls, there appears to be a selective increase in the NA content of the alpha- and beta-globulins in this disease with a slight reduction in the NA values of the albumin and gamma globulin fractions. These findings are in general agreement with those of Greenspan[39] who also reported a shift in serum glycoprotein values from one protein fraction to another in pathologic sera.

From the viewpoint of clinical investigation or routine laboratory analysis of neurological disorders, it is unfortunate that this elegant and informative technique is neither practical nor does it provide good quantitative data. However, the demonstration that there is a comparatively larger amount of NA present in the serum globulin fraction, as compared to albumin in the sera of AFI patients suggested the possibility of employing a simple salt frac-

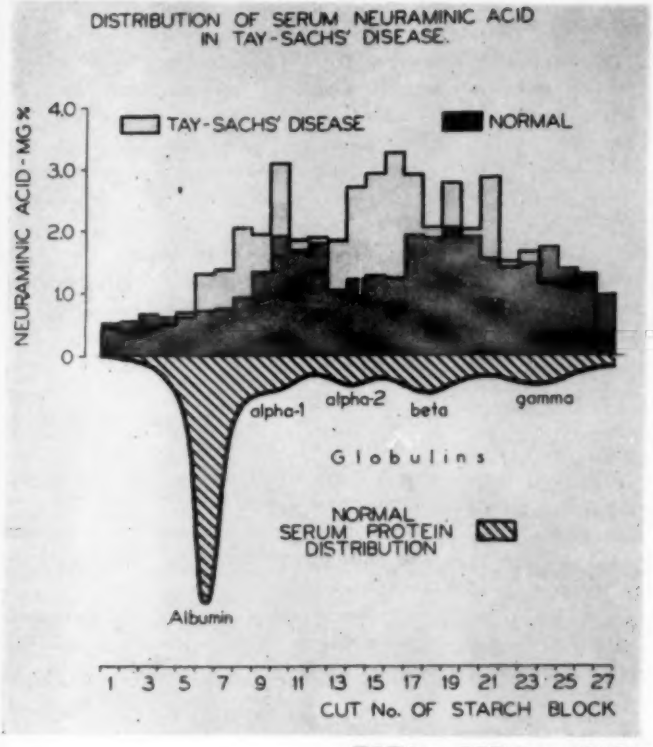


Fig. 5.—Distribution of serum neuraminic acid levels associated with various protein fractions as determined by starch block electrophoresis of a patient with amaurotic family idiocy as compared to a normal individual. (From Volk, et al., *J. Lab. & Clin. Med.* 50: 26-35, 1957.)

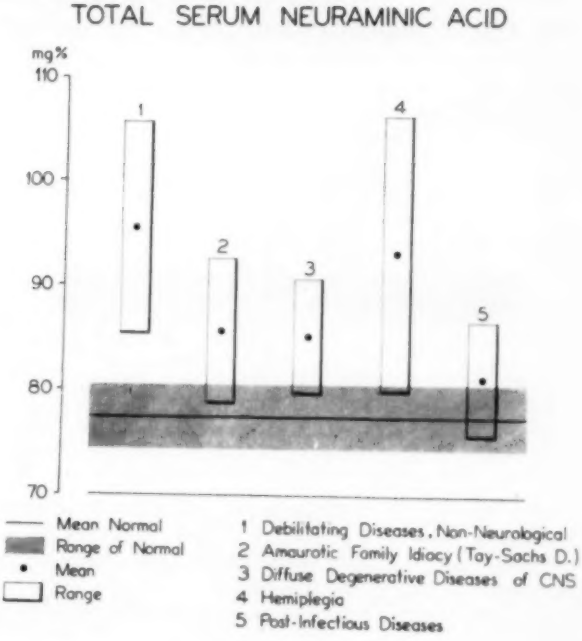


Fig. 6. — Total serum neuraminic acid values of patients in various disease groups as compared with a group of normal controls. (From Volk, et al., *J. Lab. & Clin. Med.* 50: 26-35, 1957.)

tionation procedure quantitatively separating the more soluble albumin fraction from the total globulins[40] and then releasing the NA from the precipitated globulin fraction by means of hydrolysis with trichloracetic acid and heat. The NA content of the globulin fraction was then quantitatively determined by means of the relatively specific DPA reaction.[38] The TNA was determined directly on the whole serum and the albumin NA content was then calculated by difference. As suggested by Winzler[1] a purified serum orosomucoid solution of known NA content[41] was used as a standard for the colorimetric determinations.

The TNA values obtained with this quantitative procedure for patients in various disease and control groups, are shown graphically in Figure 6. The serum TNA values are significantly elevated in Group 1 (non-neurologic debilitating diseases) and in Group 4 (hemiplegias). A lesser increase is observed in Group 2 (AFI) and Group 3 (diffuse degenerative diseases of the CNS). The data in Figure 6 illustrate the rather general nature of the increase in the serum TNA values for most diseases, non-neurologic as well as neurologic, as compared to the group of normal individuals. However, when the ratios of the serum globulin neuraminic acid/total serum neuraminic acid (GNA/TNA) are calculated for each of the five disease groups and the normal control group, the AFI patients (Group 2) become sharply delineated from the other four disease categories as illustrated in Figure 7. The serum TNA and GNA results obtained with this simple salt fractionation method confirms those found with the more elaborate starch block electrophoresis indicating a selective increase in NA-rich alpha- and beta-glycoproteins (or glycolipids) in AFI as compared to normal sera, or sera of patients with most other diseases, as determined by group statistics.

C. Cerebrospinal Fluid Neuraminic Acid Distribution in Neurologic Diseases.—The positive serum findings discussed above, as

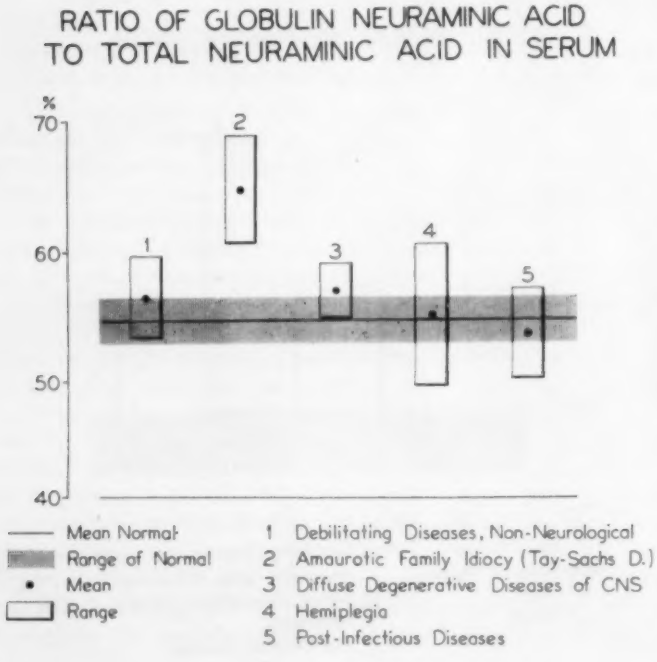


Fig. 7.—Ratio values of serum globulin neuraminic acid/total serum neuraminic acid of patients in various disease groups as compared with a group of normal controls. (From Volk, et al., *J. Lab. & Clin. Med.* 50: 26-35, 1957.)

well as the known increased synthesis of NA containing brain gangliosides in the gray matter of patients with AFI and related disorders [9,12] led naturally to an investigation of the role which the NA content of the CSF may play in this relationship. It should be stressed at this point that while NA is invariably found as part of larger complexes in either serum or tissues, e. g., glycoproteins, glycolipids, gangliosides, etc., it has been reported by Uzman and Rumley [27] to be present in the CSF mainly in a "free" form (80%), i. e., not bound to proteins. The quantitative determination of the TNA and PBNA of individual CSF specimens has been handicapped by the lack of suitable procedures requiring less than 2 ml. per determination and still relatively specific. Such a procedure [35] was developed in this laboratory using a modification of the DPA method already published for serum.[33] Quantitative separation of the TNA of the CSF into a free neuraminic acid fraction (FNA) and a protein-bound fraction (PBNA) was achieved by means of the addition of alcohol and heat (65° C for 20 minutes) as suggested by Sudhof et al.[43] Alcohol was then removed from the protein precipitate by means of heating in a boiling water bath, the NA separated from the protein by means of acid (TCA) hydrolysis and the amount present was determined quantitatively with the DPA reaction. The TNA content of the CSF was determined on a separate aliquot of the CSF by direct TCA hydrolysis of the NA from the total protein (TP) followed by application of the DPA reaction to an aliquot of the supernatant fluid. The FNA is then calculated as the difference between the TNA and the PBNA.

The results obtained with this procedure for the CSF of 42 patients with various neurological diseases, as compared to a control group of 15 presumed normal subjects, were analyzed by the techniques of group statistics [36] and are given in Table 1. The AFI group (Group C) shows only a slightly elevated TP as compared to the control group (Group E). The cases in Group D

TABLE 1.—*Group Statistical Data of Cerebrospinal Fluid Proteins and Neuraminic Acid Values in 42 Patients with Various Neurologic Diseases as Compared with a Control Group of 15 Presumed Normal Subjects*

Disease Groups	Total Protein, Mg. %	Total Neuraminic Acid, Mg. %	Protein Bound Neuraminic Acid, Mg. %	Free Neuraminic Acid, Mg. %	Total Neuraminic Acid / Total Protein, %	Protein Bound Neuraminic Acid / Total Neuraminic Acid, %
A. Vascular Diseases of Central Nervous system. (Parkinsonism, cerebrovascular disorders, etc.) (11 cases)	*34.7±3.8	6.7±0.4	1.4±0.08	5.3±0.4	20.9±1.8	20.9±1.2
	†24.1–45.3	5.6–7.8	1.2–1.6	4.2–6.4	15.9–25.9	17.5–24.3
	‡ N. S.	<0.01	N. S.	N. S.	N. S.	N. S.
B. Demyelinating Diseases (Multiple Sclerosis, Schilder's Disease, etc.) (9 cases)	*34.8±3.9	6.4±0.8	1.1±0.1	5.6±0.7	19.6±2.0	16.7±1.3
	†33.7–35.9	4.1–8.7	0.8–1.4	3.6–7.6	13.9–25.3	13.0–20.4
	‡ N. S.	N. S.	N. S.	N. S.	N. S.	N. S.
C. Amaurotic Family Idiocy (Tay-Sachs' Disease) (12 cases)	*37.3±3.2	3.7±0.4	0.8±0.06	3.0±0.3	10.2±0.7	21.4±1.7
	†28.3–46.3	2.6–4.8	0.6–1.0	2.2–3.8	8.2–12.2	16.6–26.2
	‡ N. S.	<.001	<.01	<.01	<.01	N. S.
D. Cerebral Degeneration—Extensive (Hydrocephalus, Niemann-Pick, Craniopharyngioma, etc.) (10 cases)	*151±22.7	8.5±1.2	2.9±0.4	5.6±1.1	6.4±1.3	36.9±4.3
	†87.2–214.8	5.1–11.9	1.8–4.0	2.5–8.7	2.8–10.0	24.7–49.1
	‡ <.01	<.01	<.01	N. S.	<.01	<.01
E. Control Group (Non-neurological Cases) (15 Cases)	*29.0±1.6	5.1±0.3	1.2±0.07	4.0±0.3	17.9±0.7	24.0±2.2
	†24.6–33.4	4.3–5.9	1.0–1.4	3.2–4.8	16.0–19.8	17.9–30.1

* Mean ± Standard Error (S. E.).
† Estimated range of population = Mean ± tx (S. E.).
‡ Probability at 0.01 level N. S. Not Significant.

(extensive cerebral degeneration) were selected mainly on the basis of their elevated TP values in order to determine whether a proportional relationship existed between the TP and TNA content of CSF. As noted in columns 2 to 5 of Table 1, there is only a slight increase in the TNA, PBNA and FNA in Group D over the control values as compared to the markedly elevated TP values of the CSF. For the AFI cases (Group C) where there is only a slight increase in the TP, the TNA, PBNA and FNA, all show a significant *decrease* as compared to the control group. The AFI group also can be distinguished from other groups with relatively normal TP values, in that the TNA/TP ratio is significantly decreased. The values for the PBNA/TNA ratio given in column 7 show that 70 to 82% of the TNA is in a free or unbound form which confirms the previous findings of Uzman and Rumley.[27] Since the PBNA/TNA ratio does not change significantly for the AFI group, the decrease in the TNA must be due mainly to the decrease in the FNA in this disease. According to these authors,[27] such a finding would indicate either an increased synthesis of the FNA into more complex structures such as may occur during formation of the gangliosides in the brain tissue of cases with AFI. Alternatively, it may mean a possible decrease in the activity of certain enzymes, such as neuraminidase,[44] which is responsible for the splitting of the glycosidic linkage joining the terminal NA to the residual polysaccharides or polylipids. The presence of such enzymes have yet to be demonstrated in human biological fluids.

Conversely, in cases with elevated TP of Group D, the increase in the TNA is due mainly to an increase in the PBNA fraction of the CSF which Uzman and Rumley[27] attribute to the increase in the serum PBNA complexes permeating the blood brain barrier in these types of degenerative diseases.

The individual CSF values for the various cases shown in Table 2 again illustrate that there is no direct relationship between the TP and the TNA content of this fluid. The data also suggest that there is a wide range of TNA/TP values in various neurologic conditions, i. e., from a low of 3.0% in Niemann-Pick's disease to a high of 28.8% in a case of amyotrophic lateral sclerosis. In general the most characteristic NA finding in the CSF and serum of cases

TABLE 2.—*Individual Data of Both Cerebrospinal Fluid and Serum Neuraminic Acid Distribution Levels in Patients with Various Neurological Diseases*

	Cerebrospinal Fluid					Serum		
Patient and Diagnosis	TP Mg. %	TNA Mg. %	PBNA Mg. %	FNA Mg. %	TNA / TP%	TNA Mg. %	GNA Mg. %	GNA / TN%
1 Cerebral Palsy	34.5	4.8	0.8	4.0	13.9	81.7	56.8	69.5
2 Cerebral Vascular Disease with Hemiplegia	47.7	6.7	1.5	5.2	14.0	76.8	43.6	56.7
3 Post-Poliomyelitis	23.9	5.4	1.8	3.6	22.6	87.3	54.5	62.4
4 Amyotrophic Lateral Sclerosis	43.0	12.4	1.7	10.7	28.8	--	--	--
5 Multiple Sclerosis	44.2	7.2	1.0	6.2	16.4	82.4	46.7	56.7
6 Spina Bifida	101.4	4.9	2.8	2.1	4.9	--	--	--
7 Hydrocephalus, Congenital	99.0	11.6	4.8	6.8	11.7	--	--	--
8 Craniopharyngioma	138.0	4.8	2.6	2.2	3.5	--	--	--
9 Schilder's Disease	55.5	4.5	0.9	3.6	8.1	97.6	67.4	69.0
10 Niemann-Pick's Disease	213.0	5.9	2.4	3.5	3.0	61.4	36.5	69.0
11 AFI, Infantile	25.0	3.2	0.8	2.4	12.7	101.0	67.4	67.0
12 AFI, Infantile	42.8	5.2	0.8	4.4	12.1	101.0	67.8	67.1
13 AFI, Infantile	47.5	4.1	1.1	3.0	8.6	107.0	74.6	69.7
14 AFI, Infantile	45.2	3.4	0.9	2.5	7.6	108.2	68.0	63.0
15 AFI, Juvenile	30.9	5.4	0.7	4.7	17.3	68.8	43.1	62.7
16 Normal	42.8	4.8	1.3	3.5	11.1	61.7	33.7	54.6

T. P. = Total protein. TNA = Total neuraminic acid. PBNA = Protein bound neuraminic acid. FNA = Free neuraminic acid. TNA = globulin neuraminic acid.

with AFI, as shown in Table 1, is a marked decrease in PBNA values of the CSF and an increase in serum GNA/TNA ratio values.

Summary and Conclusions

The widespread presence of neuraminic acid (NA) derivatives and related compounds, e. g., sialic acid, hematominic acid, lactaminic acid, etc., in association with proteins or lipids indicates them to be substances of considerable physiological importance. NA-containing lipids (gangliosides) were found to be markedly increased in the gray matter in cases of amaurotic family idiocy (AFI).

A simplified method was developed for the quantitative determination of the NA content of serum and its globulin fraction utilizing salt fractionation followed by the relatively specific diphenylamine colorimetric reaction.

The method was employed to investiagte the quantitative distribution within the albumin and globulin serum protein fractions of 37 normal subjects and 111 patients with neurologic and non-neurologic diseases. The sera derived from patients were divided into 5 generic disease categories for purposes of group statistics. One of these groups (Group 2) consisted of 8 patients with AFI on whom 36 analyses were performed.

The total serum neuraminic acid (TNA) was found to be elevated in all the disease groups with the highest mean value noted in the non-neurologic debilitating diseases (e. g., rheumatic fever, rheumatoid arthritis, malignant neoplasms, etc.). The globulin neuraminic acid/total neuraminic acid ratio was found to be the best statistical indicator for biochemically distinguishing AFI from other disease groups. That the increase in this ratio represents a relative shift of NA-rich glycoproteins or glycolipids from the albumin to the globulin protein fraction was verified by means of starch block electrophoresis of normal and AFI sera.

Methods were also developed for the quantitative determination of the total neuraminic acid (TNA), protein-bound neuraminic acid (PBNA) and free neuraminic acid content (FNA) of CSF requiring but 1.5 ml. of fluid. The method is based on the separation of PBNA from FNA by means of alcohol precipitation and heat.

Individual values were obtained upon the CSF of 42 patients with various neurologic diseases as well as for a control group of 15 non-neurologic cases with normal total protein (TP) values. Contrary to the findings in serum or tissues, 60-80% of the TNA is present in the free form (i. e., unbound to protein).

There is no direct relationship between TP of the CSF and its NA content. A relative increase in the PBNA, as a percentage of the TNA (Group D, Extensive Cerebral Degeneration with High TP) is interpreted as being due to the passage of NA-rich serum components across the injured blood-brain barrier. Contrariwise, a relative decrease in FNA, as a percentage of the TNA (Group C, AFI) is interpreted as representing increased synthesis of NA-containing gangliosides in brain gray matter in AFI.

The distinctive serum (increased GNA/TNA) and CSF (low PBNA, low TNA/TP with slightly increased TP) findings in AFI as compared to most other neurological diseases are based upon group statistics and should be applied with caution to the diagnosis of individual cases. It is hoped, however, that such findings may help to provide a clearer insight into the mechanisms by which normal or abnormal gangliosides are formed in AFI and other lipidoses.

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Appendix

Determination of Serum Neuraminic Acid*

A. Reagents and Apparatus

1. Trichloracetic acid (TCA) 10 per cent, 6 per cent, and 5 per cent solutions.

2. Sodium sulfite. 28 per cent solution.

3. Ether-span. Filter mixture of 1.0 ml. of Span 20† with 99 ml. of ether (reagent grade) through Whatman No. 1 filter paper and make up to the original volume with ether. Keep refrigerated when not in use.

4. Sodium chloride. 0.85 per cent solution.

5. Diphenylamine (DPA) Reagent. 1.0 per cent. Recrystallize diphenylamine‡ twice from hot ethanol and dissolve 1.0 gm. in mixture of 90 ml. of glacial acetic acid and 10 ml. of concentrated sulfuric acid.

6. Neuraminic acid standard. 0.20 mg. per milliliter. Obtain a purified orosomucoid protein sample from outdated blood bank plasma by the procedure of Weimer, Mehl, and Winzler. This preparation has a reported neuraminic acid content of about 11.2 per cent and was checked against a reference sample.§ Dissolve 17.8 mg. of the orosomucoid preparation in 10 ml. distilled water solution. This solution was stable over a 2-week period when frozen.

7. Evaporation caps to fit 15×150 mm. Pyrex tubes.¶

B. Procedure for Total Neuraminic Acid Content (TNA) of Serum

Pipette, in duplicate, 0.10 ml. of each serum sample into 15×150 mm. test tubes. Add 5 per cent TCA to each tube, with constant shaking to a final volume of 5.0 ml. Pipette, in duplicate, 0.4 and 0.8 ml. aliquots of the orosomucoid standard solution, dilute with distilled water to 2.5 ml., then add 2.5 ml. of 10 per cent TCA and mix by inversion. Cover each tube with an evaporation cap and heat in a boiling water bath for exactly 15 minutes. Cool tubes in cold tap water and centrifuge at 3,000 r. p. m. for 15 minutes. Pour off supernatant fluid into a small test tube; recentrifuge and decant into another tube if fluid is not perfectly clear.

Pipette 2.0 ml. aliquots of the clear fluids into 15×150 mm. test tubes. Add 4.0 ml. of DPA reagent to each tube and mix by tapping the bottom of the tube with a finger. Prepare a reagent blank by adding 2.0 ml. of 5 per cent TCA to 4.0 ml. of DPA reagent. Cover each tube with an evaporation cap and heat in a boiling water bath for exactly 30 minutes. Cool to room temperature in a cold water bath and read optical density values in a spectrophotometer at 530 mμ with the reagent blank set at zero.

* Atlas Powder Co., Wilmington, Del.

† Fisher Scientific Co., New York, N. Y.

‡ This sample of known neuraminic acid content was generously provided for our use by Dr. R. J. Winzler of the University of Illinois College of Medicine.

§ Obtainable from Kopp Laboratory Supplies, 70-13 35th Rd., Queens 72, N. Y.

Calculations: Neuraminic Acid (mg. per 100 ml. serum) $= \frac{\text{O. D. unk}}{\text{O. D. stnd}} \times \text{Conc. stnd} \frac{\times 100}{0.04}$

Since color reaction deviates from Beer's Law, standard used in the calculation should be that which is closest to the unknown.

C. Procedure for Neuraminic Acid Content of Globulin Fraction (GNA) of Serum

Pipette, in duplicate, 6.0 ml. of 28 per cent sodium sulfite into 15×150 mm. test tubes and add 0.25 ml. of serum. Mix by inverting tubes once and then overlay with 3 to 4 ml. of ether-span. Mix 20 times by gentle inversion. (The tube is turned upside down and then back to its original position for one inversion.) Centrifuge for 10 minutes at 2,000 r. p. m. A button containing the total globulins is formed at the interface. By careful tilting, the button can be disengaged from the side of the tube. A long thin capillary is inserted at an angle along the underside of the tube to the bottom and the supernatant fluid drawn off by means of gentle water suction. By means of a small polyethylene wash bottle, let a fine stream of water flow gently down the sides of the tube without disturbing the globulin button. Remove the wash water with the capillary pipette by gentle suction. Repeat this washing procedure twice more in order to remove as much of the albumin-sodium sulfite solution as is possible.

Pipette 1.0 ml. of 0.85 per cent sodium chloride into each tube and mix by tapping with finger in order to suspend the globulin fraction. Add 4.0 ml. of 6 per cent TCA, mix contents, cover with evaporation caps and place in boiling water bath for exactly 15 minutes. The remainder of this procedure is exactly as described in paragraph B for total serum neuraminic acid.

Calculations: Neuraminic Acid (mg. per 100 ml. serum) $= \frac{\text{O. D. unk}}{\text{O. D. stnd}} \times \text{Conc. stnd} \frac{\times 100}{0.1}$

Use standard value closest to that of the unknown in calculating the results.

Normal Values

Serum values obtained with the procedure for 37 normal individuals (26 adults and 11 children) were as follows: total serum neuraminic acid, 77.4±7.3 (S. D.) mg. per cent; globulin neuraminic acid, 42.3±4.6 (S. D.) mg. per cent; globulin neuraminic acid: total neuraminic acid, 54.7±4.3 (S. D.) per cent; globulin neuraminic acid: globulin protein, 1.46±1.6 (S. D.) per cent. These normal serum results are somewhat higher than those obtained by Winzler.

Determination of Cerebrospinal Fluid Neuraminic Acid and Total Protein[35]

1. Sodium Chloride: 0.85% solution.
2. Sodium Hydroxide: 10 N solution.
3. Biuret Reagent (Modified after Gornall, et al.[39]): In a liter volumetric flask containing about 500 ml. of distilled water, place 3.0 g. of $CuSO_4.7H_2O$, 12 g. of sodium potassium tartrate (Rochelle salt) and shake until dissolved. Add with shaking 600 ml. of 2.5N NaOH. Add 2 g. KI, shake until dissolved and dilute to volume with distilled water.
4. Total Protein Standard: Reconstitute a prepared serum standard, e. g., Versatol,‖ with 5.00 ml. of a solution composed of 10% NaCl plus 1.0% of disodium ethylenediamine tetracetic acid. Refer to manufacturer's chart for protein content. Dilute 1.00 ml. to 100 ml. with 0.85% NaCl in a volumetric flask. Keep refrigerated when not in use and discard when cloudy.
5. Trichloracetic Acid (TCA): 5.0, 7.5 and 10% solutions were prepared by dilution from a 90% stock solution.
6. Diphenylamine (DPA) Reagent. 1.0%. Recrystallize diphenylamine twice from hot ethanol to remove impurities. Dissolve 1.0 g. in a mixture of 90 ml. of glacial acetic acid plus 10 ml. of concentrated sulfuric acid.
7. Neuraminic Acid Standard. A purified orosomucoid protein sample was prepared from outdated blood bank plasma by the procedure by Weimer, et al. This preparation has a reported NA content of about 11.2% and was checked against a reference sample supplied by Dr. R. J. Winzler. For a 10 mg % NA standard, dissolve 8.9 mg. of the orosomucoid preparation (11.2% NA) in 10 ml. of distilled water. 5.0 mg. % and 2.5 mg. % NA standards were then prepared by dilution from this solution. The 10 mg. % standard solution will remain stable for about one week if kept frozen between runs.
8. Absolute ethyl alcohol.
9. Evaporation caps to fit 13×125 mm. Pyrex tubes.¶

B. Determination of Total Protein Content of Cerebrospinal Fluid

Pipette 1.00 ml. of CSF samples into a small test tube calibrated at 2.0 ml. Add 1.0 ml. of 10% TCA to each tube, and let stand at room temperature for 15 minutes. Centrifuge precipitates at 3,000 r. p. m. for 10 minutes. Decant off supernatant fluids and dissolve precipitates in one drop of 10N NaOH by shaking tubes until precipitates are dissolved. Add 1.00 ml. of biuret reagent, and dilute to 2.00 ml. mark with 0.85% NaCl solution. Mix well, let stand for 20 minutes and read in a spectrophotometer at 530 mμ or in a Klett-Summerson colorimeter, in a flat bottom colorimeter tube, with a #54 filter against a saline blank (1.00 ml. 0.85%

‖ Versatol is sold by Warner-Chilcott Co., Morris Plains, N. J.

¶ Obtainable from Kopp Laboratory Supplies, 70-13 35th Rd., Queens 72, N. Y.

NaCl plus 1.00 ml. biuret reagent) which has been set at zero. The TP standards are run simultaneously and exactly in the same manner as were the CSF samples.

Calculation:

$$\text{TP (CSF) mg. \%} = \frac{\text{O. D. unk}}{\text{O. D. stnd}} \times \text{TP Standard (mg. \%)}$$

C. Determination of Total Neuraminic Acid (TNA) Content of Cerebrospinal Fluid

Pipette 0.50 ml. of CSF samples into 15 ml. centrifuge tubes. Add 1.0 ml. of 7.5% TCA to each tube. Mix and cover tubes with glass evaporation caps. Heat tubes in a boiling water bath for 15 minutes. Cool tubes in cold tap water and centrifuge precipitates # at 3,000 r. p. m. for 10 minutes. Pipette 1.00 ml. aliquots of the supernatant fluid into 13×125 mm. test tubes. Add 2.00 ml. of the DPA reagent and mix. Cover each tube with an evaporation cap and place in a boiling water bath for 30 minutes. Cool to room temperature in a cold water bath. A reagent blank (0.50 ml. water) and 0.5 ml. aliquots of 5 mg. % and 10 mg. % NA standards, in duplicate, are carried through exactly the same procedure as are the CSF samples. The optical density values of the unknowns and standards are then read in a spectrophotometer at 530 mμ (or in a Klett photoelectric colorimeter with a No. 54 filter) against a reagent blank set at zero.

Calculations:

$$\text{Total Neuraminic Acid (CSF) mg. \%} = \frac{\text{O. D. unk}}{\text{O. D. stnd}} \times 10.0 \text{ (or } 5.0)$$

To conserve CSF, the dried precipitates obtained in this step can be dissolved in one drop of 10N NaOH with shaking and the remainder of the procedure is carried out as described above except that the results are multiplied by 2.

Since the color reaction deviates from Beer's Law, the standard used in making the calculations should be that which is closest to the unknown.

D. Determination of Protein Bound Neuraminic Acid Content of Cerebrospinal Fluid

Into 15 ml. centrifuge tubes pipette 1.00 ml. aliquots of the CSF and add to each tube 4.0 ml. of absolute alcohol. Mix contents and place tubes in a 65 C water bath for 20 minutes. Cool tubes to room temperature in cold tap water and centrifuge at 3,000 r. p. m. for 10 minutes. Decant off supernatant fluid as completely as possible and break up precipitates by tapping bottom of the tube. Dry the precipitates by placing tubes in a boiling water bath until no alcohol odor can be detected. Add 1.5 ml. of 5.0% TCA to each tube containing the alcohol precipitates. Pipette, in duplicate, into 15 ml. centrifuge tubes 0.50 ml. of distilled water (for reagent blank) and 0.5 ml. of 2.5 mg. % and 5.0 mg. % NA standards. Add 1.0 ml. of 7.5% TCA to blank and standard tubes *only*. Mix and cover mouths of all tubes with evaporation caps. Heat all tubes in a boiling water bath for exactly 15 minutes. Cool tubes to room temperature in cold tap water and centrifuge at 3,000 r. p. m. for 10 minutes. Pipette 1.00 ml. aliquots of the supernatant fluid into 13×125 mm. test tubes. Add 2.00 ml. of DPA reagent and mix. The remainder of the procedure is exactly as described for the TNA determination in paragraph C above.

$$\text{Protein Bound Neuraminic Acid (mg. \%)} = \frac{\text{O. D. unk}}{\text{O. D. stnd}} \times 2.5 \text{ (or } 1.25)$$

Normal Values

The normal values with these procedures are listed in Table 1 (Group E—Control Group).

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